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POTENZIALE COINVOLGIMENTO DELL'NGF NELL'INFIAMMAZIONE E NEL RIMODELLAMENTO TISSUTALE DELLA SUPERFICIE OCULARE

Barbara Stampachiacchiere

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Docente Guida: Prof. Stefano Bonini

Coordinatore: Prof. Enrico Garaci

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Capitolo 1

NGF

1.1 Nerve Growth Factor (NGF):

Le neurotrofine (NTs), polipeptidi che supportano la crescita, la differenziazione, la sopravvivenza, l'apoptosi e la modulazione sinaptica delle cellule nervose, furono inizialmente scoperte nel sistema nervoso centrale. Il primo e il meglio caratterizzato tra le NTs è il nerve growth factor (NGF), il quale agisce non solo a livello del sistema nervoso ma ha effetti fino ai sistemi: immunitario, endocrino e visivo.

La scoperta dell'NGF, nel 1950 da Rita Levi-Montalcini, portò alla formulazione dell'ipotesi dei "fattori neurotrofici" che si basava sull'idea che durante lo sviluppo i neuroni competessero tra loro per i limitati supplementi neurotrofici presenti nel tessuto bersaglio. Stava quindi alla selezione naturale decidere quale neurone sarebbe morto o sopravvissuto. Oggi, dopo molti anni di studi, si è compreso che questi meccanismi sono di gran lunga più complessi di quanto si pensasse 60 anni fa e di essere ancora lontani dal conoscerli a fondo. La scoperta dell'NGF non limitato solo al sistema nervoso ma avente anche un ruolo ponte con altri compartimenti è stato fondamentale nello studio dei meccanismi di interazione tra i diversi sistemi cellulari.

La maggior conoscenza dei meccanismi di regolazione e d'azione di queste NTs sarebbe

di aiuto nella comprensione e nella prevenzione delle disfunzioni che sono alla base di molte patologie neurologiche e non solo.

1.1.1 storia dell'NGF.

L'NGF è stato scoperto durante una serie di esperimenti necessari per definire le interazioni tra lo sviluppo dei neuroni e i loro tessuti target. Nel 1950, nell'istituto di Zoologia della Washimgton University, E.D. Bueker ebbe l'intuizione di impiantare un tumore murino di origine connettivale (noto come Sarcoma 180) nella parete addominale di embrioni di pollo, al 3° giorno di sviluppo. Dopo pochi giorni dall'impianto, si vide che i gangli sensitivi adiacenti al tumore erano andati incontro ad un considerevole aumento di sviluppo. I tessuti neoplastici erano stati totalmente invasi da fibre nervose emergenti e le viscere dell'embrione erano state raggiunte da un grande numero di fibre simpatiche molto più velocemente rispetto agli embrioni di controllo. Successivamente, si ottennero gli stessi risultati trapiantando frammenti del Sarcoma 180 o del Sarcoma 37 (che possiedono caratteristiche molto simili tra loro) nella membrana, molto vascolarizzata, corion-allantoidea dell'uovo di pollo che alimentava sia il tessuto in normale sviluppo, che quello neoplastico [1]. La prima ipotesi fu quella che il tumore rilasciasse un fattore chimico diffusibile capace di indurre la crescita gangliare, la produzione e la ramificazione atipica delle fibre nervose [2]. Il misterioso fattore X, capace di svolgere un'azione stimolante sulle cellule nervose sensitive e simpatiche dell'embrione di pollo, agiva per via umorale [1]. La prima domanda che Cohen e Levi-Montalcini si posero fu riguardo alla natura chimica del fattore X: "acido nucleico o proteina?". Come prima cosa trattarono l'estratto di cellule tumorali con veleno di serpente, ricco di fosfodiesterasi, per degradare gli acidi nucleici [2]. Ciò permise di scartare la prima ipotesi sulla natura nucleare del fattore, dal momento che piccole aliquote di veleno aggiunte alla frazione tumorale aumentava enormemente l'effetto di crescita del tumore stesso. Durante questi esperimenti

i ricercatori notarono un episodio sorprendente, ossia che aggiungendo piccole quantità di veleno ad un mezzo di coltura, privo della frazione tumorale, si otteneva lo stesso alone intensissimo di fibre nervose provenienti dai gangli sensitivi e simpatici del pollo. Questo stava a significare che anche il veleno di serpente doveva contenere il fattore X. Nel 1959 Cohen riuscì purificare la proteina responsabile dal veleno di serpente a cui venne dato il nome di NGF (fattore di crescita neuronale) [3]. Nello stesso anno, si scoprì che le ghiandole salivari di topo erano un'eccellente fonte per la purificazione dell'NGF, dal momento che esse ne contengono una quantità circa diecimila volte superiore a quella del sarcoma di topo e circa dieci volte maggiore di quella presente nel veleno di serpente [3, 4]. Dalla scoperta dell'NGF ad oggi, sono stati fatti numerosi studi che hanno portato all'identificazione della sua struttura chimica, dei siti di sintesi, delle cellule bersaglio e dei meccanismi di azione. Dopo l'NGF sono stati identificati altri fattori di crescita tra i quali il Brain Derived Neurotrophic Factor (BDNF), la Neurotrofina 3 (NT-3), la Neurotrofina 4 (NT-4) e la Neurotrofina 5 (NT-5). Questi fattori di crescita mostrano fra loro una considerevole omologia sia a livello strutturale che a livello dei siti recettoriali [5].

Gli studi degli ultimi anni hanno messo in luce la plasticità dell'NGF rispetto le altre NTs, la quale viene definita una modulatrice delle funzioni Neuro-Immuno-Endocrine, in particolare nella regolazione dell'omeostasi [6].

1.1.2 Struttura.

Le ghiandole sottomandibolari di topo sono la prima fonte di purificazione dell'NGF [7]. L'NGF rilasciato dal tessuto ghiandolare e purificato secondo il metodo di Varon et al. (1967) è un complesso molecolare, costituito da tre subunità dimeriche: α β e γ, ($\alpha 2 \beta 2 \gamma 2$), di 140 KDa ed un coefficiente di sedimentazione pari a 7S. La subunità β, chiamata anche βNGF, rappresenta la forma attiva dell'NGF (118 aa.; P.M. 13 KDa; coefficiente di sedimentazione pari a 2,5S) [8]. La subunità γ è un'arginina peptidasi (P.M. 26 KDa), mentre non è stato

ancora del tutto chiarito quale sia il ruolo della subunità α (P.M. 26000 Dalton) [7]. Le singole catene peptidiche sono stabilizzati da legami covalenti (tre ponti disolfuro ognuna), diversamente i complessi dimerici sono mantenuti da legami non covalenti. Le due catene della subunità β vengono sintetizzate a partire da un precursore più grande, detto pro-NGF, che viene scisso nel dimero attivo attraverso l'azione proteasica delle subunità γ. Svolta la loro azione proteasica, le subunità γ restano legate al dimero βNGF, al quale si aggiungono anche le subunità α conferendo al βNGF cosi complessato una protezione da ulteriori azioni proteolitiche indesiderate [10]. L'analisi cristallografica ha rilevato che il βNGF è costituito da tre coppie antiparallele di filamenti, con struttura secondaria di tipo β, in grado di formare una superficie piana lungo la quale si associano le due catene per dare il dimero attivo [11]. Inoltre, queste catene sono caratterizzate da quattro regioni "loop" in cui sono localizzati molti amminoacidi variabili ai quali, probabilmente, è legata la specificità di riconoscimento da parte del recettore [5, 11].

1.1.3 Gene.

L'intera sequenza genetica dell'NGF fu inizialmente definita nel topo. Il gene, in singola copia, è situato sul cromosoma 1, ed è costituito all'estremità 3' dall'esone IV che codifica l'intera sequenza per la catena del βNGF, ed all'estremità 5' da una serie di piccoli esoni, [12]. Il confronto dell'organizzazione fra il gene murino e la porzione nota del gene umano dell'NGF ha rivelato una perfetta corrispondenza delle dimensioni degli esoni all'estremità 5' e della localizzazione degli introni [12, 13]. Il gene murino trascrive tre proteine precursori dell'NGF, differenti a livello dell'estremità NH2-terminale. Ciò potrebbe esser dovuto o all'utilizzo di differenti siti di inizio di trascrizione, o ad alternativi processi di splicing dell'esone II. Entrambi i meccanismi permetterebbero di produrre, da un singolo gene, peptidi simili ma con una diversa localizzazione cellulare. Dal trascritto più corto probabilmente si originerebbe la forma secretoria dell'NGF, mentre dal trascritto di maggiore lunghezza la forma di membrana,

capace di interagire con recettori specifici localizzati sulla membrana delle cellule contigue sensibili all'azione dell'NGF [140]. Studi di clonaggio molecolare hanno mostrato una forte omologia di sequenza dell'NGF di diverse specie (topo, uomo, pollo, bue, maiale, cobra e Xenopus) che va tra il 66% ed il 98%, indice del fatto che questa molecola è rimasta quasi inalterata nel corso dell'evoluzione. L'alta omologia di sequenza presente anche tra l'NGF e le altre NTs suggerisce un'appartenenza ad una stessa famiglia genica per tutti i fattori neurotrofici. Su ciò si sono realizzati degli alberi genealogici con un precursore comune, la pro-insulina umana, dalla quale, per i processi di duplicazione e divergenza genica, si suppone siano derivate le differenti forme di neurotrofine attualmente note [15].

1.1.4 Sorgenti dell'NGF.

Dalla scoperta dell'NGF fino ad oggi sono stati individuati molti tessuti in grado di produrre e/o accumulare NGF, sia nei vertebrati inferiori che superiori. L'NGF è sintetizzato e rilasciato in quantità variabili da differenti fonti animali, tra cui alcuni tumori maligni di topo [1], le ghiandole velenifere degli ofidi[3], la ghiandola sottomascellare di topo maschio [16], la prostata di cavia [17, 18], i testicoli di mammifero [18], le parotidi e la tiroide di ratto [19], e la placenta umana [20]. Attualmente la maggior fonte di NGF, utilizzata per la purificazione del fattore, è rappresentata dalle ghiandole sottomandibolari di topo. La ghiandola di topo è costituita da due diverse componenti: gli acini, contenenti mucopolisaccaridi ed i tubuli convoluti. In questi ultimi, caratterizzati da dimorfismo sessuale, oltre ad accumularsi gli enzimi idrolitici, viene sintetizzato ed accumulato l'NGF. In particolare, lNGF è assente nelle ghiandole dei topi neonati, nei quali i tubuli non sono ancora differenziati; comincia ad apparire nella pubertà e subisce un aumento progressivo negli stadi successivi di sviluppo per raggiungere, infine un plateau nel maschio adulto. Queste osservazioni, correlate al fatto che la concentrazione dell'NGF nelle ghiandole è dieci volte più alta nei topi maschi che nelle femmine, hanno fatto ipotizzare che la sintesi ed il rilascio di NGF siano modulati

dall'azione dell'ormone maschile testosterone [16]. Infatti l'iniezione di testosterone in femmine di topo aumenta di circa dieci volte il contenuto dell'NGF nelle loro ghiandole salivari, mentre la castrazione dei topi maschi comporta una netta diminuizione dei livelli dell'NGF salivare [7, 21]. Studi compiuti da Aloe e Levi-Montalcini (1979) sugli effetti della tiroxina e del testosterone sulla differenziazione dei tubuli convoluti delle ghiandole salivari e sul contenuto dell'NGF durante le fasi di sviluppo post-natale del topo, hanno dimostrato che entrambi questi ormoni influenzano la sintesi e l'accumolo dell'NGF nelle ghiandole salivari e che durante la fase prepuberale, gli effetti della tiroxina sul differenziamento dei tubuli convoluti e sulla produzione dell'NGF sono molto più marcati di quelli del testosterone. Studi di correlazione tra gli ormoni tiroidei e l'NGF sono stati condotti anche a livello del Sistema Nervoso Centrale (SNC) ed è stato dimostrato che, nei topi neonati ed in quelli adulti, i livelli dell'NGF sono linearmente correlati con i livelli sierici degli ormoni tiroidei, tiroxina (T4) e di 3-idrossitironina (T3) [22]. Inoltre nel 1986 Aloe e Levi-Montalcini hanno osservato che le lotte intraspecifiche, indotte da un isolamento di 6-8 settimane, stimolano il rilascio massiccio dell'NGF nel sangue di topi maschi adulti. Attraverso studi biologici, radioimmunologici ed immunoistochimici, gli stessi autori hanno dimostrato che l'NGF è rilasciato nel sangue dopo pochi minuti dal combattimento e raggiunge un valore massimo dopo tre-quattro ore [23]. Poichè le lotte intraspecifiche rappresentano un noto modello di stress, questi studi hanno messo in evidenza una stretta correlazione fra l'NGF rilasciato e lo stress in corso. Questo rapporto tra NGF e stress, era stato messo in luce per la prima volta da Otten [24], che mediante somministrazione intravenosa di NGF in ratti adulti aveva indotto uno stimolo dell'asse Ipotalamo-Ipofisi-Surrene, la cui attivazione è nota essere strettamente associata ad una condizione stressogena. Sicuramente le ghiandole salivari hanno un ruolo attivo nel mantenere i livelli di NGF nel sangue, ma non ci sono prove a favore dell'ipotesi che esse siano essenziali o almeno necessarie per l'utilizzo di tale neurotrofina da parte delle cellule bersaglio. Una delle tante questioni che resta ancora da chiarire è se questa NTs, presente in così grandi quantità nelle ghiandoli salivari murine, possa avere anche altre funzioni, alcune

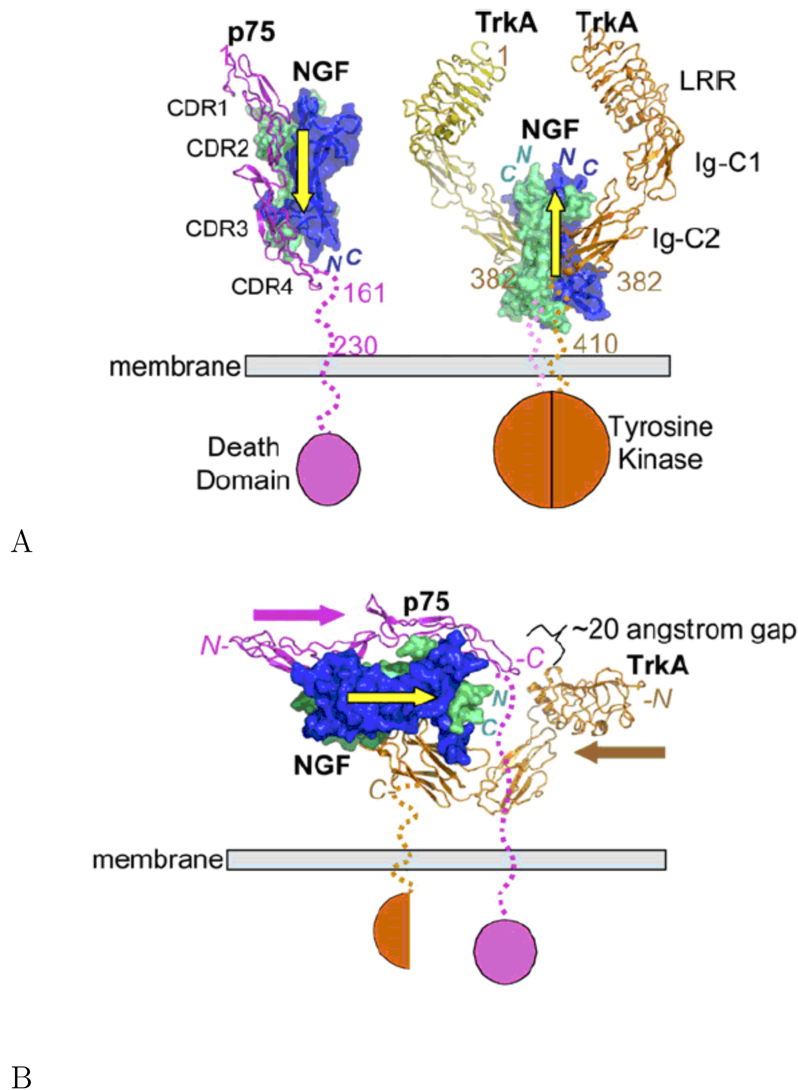
connesse con la digestione, altre collegate alla produzione ed al rilascio di sostanze tossiche nella saliva stessa [4].

1.1.5 Meccanismi di azione e traduzione del segnale.

I meccanismi successivi al legame dell'NGF alla cellula target sono tutt'ora oggetto di molteplici studi. La pluralità degli effetti dell'NGF è dovuta alla sua capacità di mediare il segnale attraverso due diversi recettori trans-membrana, una tirosin chinasi trkA^{NGFR} , che riconosce e lega in modo specifico (Kd=10-9M), ed un recettore aspecifico, il p75^{NTR} che lega indistintamente tutte le NTs (Kd=10-9M).

Il recettore storico per eccellenza dell'NGF è il p75^{NTR}, il primo ad essere isolato e caratterizzato. Nel 1984, il cDNA di p75^{NTR} fu isolato, grazie l'utilizzo di un anticorpo monoclonale capace di bloccare od alterare il legame con l'NGF in cellule PC12[1] di ratto [25]. L'analisi del cDNA, dimostò che p75^{NTR} è una proteina transmembrana di P.M. pari a 42 KD, che glicosilata raggiunge un P.M. di 75 KD [26, 27, 28]. Questo recettore, sebbene presente in molte cellule "responsive" all'azione dell'NGF, non è però dotato di attività enzimatica.

[1]Linea cellulare clonale derivata da un feocromocitoma di topo, è il modello sperimentale più utilizzato per lo studio della capacità dell'NGF di modulare espressioni fenotipiche e meccanismi molecolari.



Wehrman et al., 2007

Figura 1.1: A) Struttura dell'NGF complessato con il dominio extracellulare di p75^{NTR} (He & Garcia, 2004) e di trkA. La polarità di NGF nei due complessi è indicata in giallo, i domini intracellulare di entrambi i recettori sono disegnati schematicamente. B) rappresentazione di un complesso ternario costituito da p75/NGF/trkA nei rapporti:1/2/1, dove trkA^{NGFR} è orientato in modo antiparallelo rispetto p75^{NTR}.

L'esperimento fondamentale per la comprensione dell'attività di trasduzione del segnale dell'NGF risale al 1988, quando Maher trattò cellule PC12 con NGF e vide che ciò permetteva la rapida fosforilazione di alcune proteine non ben identificate [29]. Studi successivi misero in evidenza la presenza di un altro recettore specifico per l'NGF, di PM di 140KD,

dotato di attività tirosin-kinasica e codificato dal protooncogene trk [30]. Dopo la scoperta del trkA^{NGFR}, dotato di una traduzione del segnale all'interno della cellula altamente complesso, al p75^{NTR} fu rilegato un ruolo secondario [31]. Solo recentemente si è giunti alla conclusione che il legame ad alta affinità dell'NGF con il suo recettore specifico richiede la co-espressione dei due prodotti genici: trkA^{NGFR} e p75^{NTR}. Negli ultimi anni, l'idea che l'NGF potesse legare i due recettori contemporaneamente, per formare un super recettore, ha fomentato una vasta letteratura sull'argomento. Diversi lavori mostrano che topi knockout per p75^{NTR} richiedono concentrazioni di NGF più elevate del normale per la sopravvivenza [32, 33]. Nel 1994 Mahaded e collaboratori osservarono che la co-espressione di entrambi i recettori aumenta la velocità di associazione dell'NGF con trkA^{NGFR} [34]. Da questi ed altri lavori si attribuì al p75^{NTR} un ruolo modulatore della selettività del recettore trkA^{NGFR} , partecipando alla formazione di un super recettore specifico (Kd=10-11M) [35]. Sebbene, si è tuttavia ancora lontani dalla totale comprensione dei meccanismi attraverso i quali p75^{NTR} influenzi trkA^{NGFR} nella trasduzione del segnale. Basandosi su studi di legame, cristallografia e cross-reattività [35, 36, 37] sono stati proposti alcuni modelli in grado di spiegare il legame ad alta affinità [38, 36, 39]. In uno di questi modelli, l'NGF si legata sia ai singoli recettori trkA^{NGFR} e p75^{NTR}(omodimero), che al complesso formato dai due recettori (eterodimero) (vedi Figura 1.1AB).

I due recettori mediano una serie di segnali che vanno dalla proliferazione, differenziazione, sopravvivenza all'apoptosi dei diversi tipi cellulari appartenenti al sistema nervoso, endocrino, immunitario e visivo. Alcuni studi dimostrato che la durata e l'intensità del segnale dipendano dalla distribuzione e dal rapporto di questi sulla superficie cellulare [40].

trkA^{NGFR} ha delle caratteristiche che lo rendono strutturalmente diverso dalla famiglia dei recettori tirosinchinasi a cui appartiene. Infatti, rispetto al recettore per il Platelet-Derived Growth Factor (PDGF) e a quello dell'Epidermal Growth Factor (EGF), trkA^{NGFR} possiede:

- un inserto di 14 amminoacidi che interrompe una regione conservata del dominio cataliti-

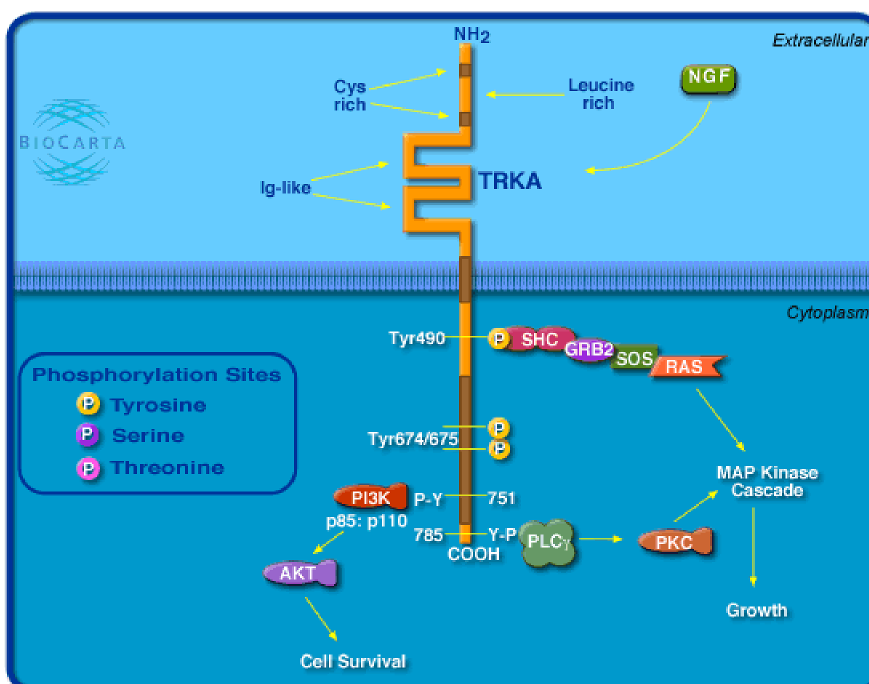
co;

- una coda -COOH terminale più corta (15 amminoacidi);
- una serie di segmenti ripetuti in tandem nel suo dominio extracellulare, ricchi di leucina, legati a due clusters di residui di cisteina ed a due domini che sono simili alle regioni C2 delle immunoglobuline permettendogli l'interazione del recettore con proteine superficiali [41].

Il segnale dell'NGF mediato via trkA^{NGFR} è determinato dall'autofosforilazione della tirosina 490 (Tyr-490), posizionata nel dominio intra-citoplasmatico del recettore al quale, di seguito, si legano proteine Shc[2], permettendogli di acquisire l'attività tirosin-chinasica [42, 43, 30] e divenendo così capace di fosforilare tutta una serie di substrati come: la fosfolipasi C (PLC 1) [44], la tirosinchinasi Src, la proteina Ras (GTP-binding) e le proteinchinasi citoplasmatiche (serina/treonina) Raf [45]. Studi sulle cellule PC12 hanno dimostrato che dopo fosforilazione della PLC 1, i livelli dell'inositolo trifosfato (IP3) e del diacilglicerolo (DAG) aumentano determinando la liberazione di calcio dai depositi cellulari e l'attivazione della protein chinasi C (PKC) [41].

L'attivazione del recettore sembra avvenire principalmente in membrane simili ai caveoli. L'NGF è trasportato all'interno della cellula nervosa mediante un meccanismo di trasporto retrogrado lungo l'assone, che avviene dalla periferia verso il nucleo. Per questo, l'internalizzazione del complesso NGF/recettore è fondamentale negli eventi di trasduzione del segnale. Questo processo inizia con un meccanismo di endocitosi e la formazione di endosomi, nei quali l'NGF è complessato con il suo recettore specifico. Alcuni passaggi coinvolti nella trasduzione del segnale del trkA^{NGFR} all'interno della cellula sono schematizzati in figura 1.2

[2]substrato di recettori ad attività tirosin-chinasica



Liisa Eisenlohr, PhD., Cell Signaling Technology

Figura 1.2: Rappresentazione schematica del legame dell'NGF al dominio di legame trkA^{NGFR} che determina un'auto-fosforilazione dello stesso recettore e l'attivazione di una serie di eventi a cascata. Le proteine che interagiscono direttamente con il dominio intracellulare di trkA^{NGFR} sono: SHC, PLC 1, e gli inibitori di apoptosi (IAPs), alcuni dei quali mostrati in figura. Il legame con trkA^{NGFR} può attivare il pathway RAS, segnale di crescita e differenzzazione, oppure ad un alternativo pathway di sopravvivenza mediante la via del fosfoinositolo 3 chinasi (PI3K).

Per molto tempo il p75^{NTR} è stato relegato in una posizione secondaria, confinato al ruolo di modulatore del segnale del trkA^{NGFR} . Tuttavia, attualmente non è più cosi, le scoperte degl'ultimi anni hanno ridato il giusto valore a questa proteina. Nel 1993, Barbacid e collaboratori osservarono che l'NGF era in grado di legare direttamente il p75^{NTR} e mediare un messaggio all'interno della cellula in assenza di attività tirosin-chinasica, attivando una serie di segnali fino ad arrivare all'apoptosi[46]. Proprio per queste caratteristiche il p75^{NTR} fu incluso nella superfamiglia dei recettori "Tumor Necrosis Factors" (TNF) [47]. Inoltre, questa omologia con i recettori TNF ha fatto pensare che i fattori ad essi associati, i TRAFs[3]

[3]Proteine coinvolte nella regolazione dell'infiammazione, nelle risposte anti-virali e nell'apoptosi. Presentano una struttura secondaria relativamente conservata, incluso il dominio che media le interazioni con con

[48] siano coinvolti nei segnali del p75^{NTR}, che riconoscono e legano a livello del suo dominio citoplasmatico [49, 50].

La maggior parte delle attività biologiche mediate dall'NGF (sopravvivenza, proliferazione, ecc) sono dovute al legame con trkA^{NGFR} che attiva una serie di proteinchinasi a catena. Diversamente, il segnale di trasduzione del p75^{NTR} include il fattore nucleare kappa B (NFkB), "c-Jun N-terminal kinase" (JNK), e l'incremento nella produzione di ceramide, responsabili della trascrizione genica o della morte programmata per apoptosi. Questi effetti opposti mediati dall'NGF (sopravvivenza e morte cellulare), apparentemente non sono mostrati dalle altre NTs, sebbene tutte leganti il p75^{NTR}.

Recentemente è stato dimostrato che il pro-NGF, isolato in diversi tessuti, sembrerebbe preferire il legame con p75^{NTR}, in associazione con il co-recettore Sortilin [4] [51, 52, 53], per promuovere segnali di apoptosi in cellule neuronali in coltura. Al contrario, le stesse cellule presentano una minima attivazione dei segnali di differenziazione e sopravvivenza mediati dal trkA^{NGFR} . Questa osservazione rafforza l'ipotesi che gli enzimi proteolitici giochino un ruolo fondamentale nel regolare l'attività biologica dell'NGF, visti i diversi effetti delle due forme della NT.

Di notevole interesse è il fatto che p75^{NTR} sia in grado di mediare segnali anche in assenza di ligandi, presubilmente quando il recettore oligomerizza o quando forma altri complessi [54, 55]. Ormai oggetto di molte attenzioni, questo recettore interessa soprattutto per la sua capacità di mediare più funzioni cellulari ed è intrigante pensare che la scelta tra queste dipenda dal contesto e dal repertorio di co-recettori presenti sulla cellula: e.g., trkA^{NGFR} [56], Nogo[5] receptor (NgR) [57], Sortilin[58], ecc. Questa caratteristica lo rende un recettore molto eclettico, a tal punto da poter mediare segnali completamenti opposti l'uno dall'altro. Nel sistema nervoso, p75^{NTR}, può promuovere[59, 60] o inibire [61](Yamashita T et al., 2002)

altre proteine regolatrici come i recettori TNF e CD40.

[4]Proteina, membro della famiglia VPS10, in grado di interagire con il p75 favorendo il legame di quest'ultimo con il pro-NGF e formando così un complesso terziario.

[5]Glicoproteina, mielina associata: è l'inibitore associato alla mielina più ampiamente studiato ed è stata identificata più di 10 anni dopo i primi esperimenti condotti da Schwab con α-IN1.

la crescita assonale, ridurre [62] o promuovere[63, 64] la morte cellulare neuronale, ed è necessario [65] o non richiesto [66] per l'inibizione della rigenerazione neuronale. Queste apparenti discrepanze non sono confinati ai soli neuroni, infatti, p75^{NTR} sembra sia inibire [67] che promuovere [59, 68] la migrazione delle cellule di Schwann durante lo sviluppo.

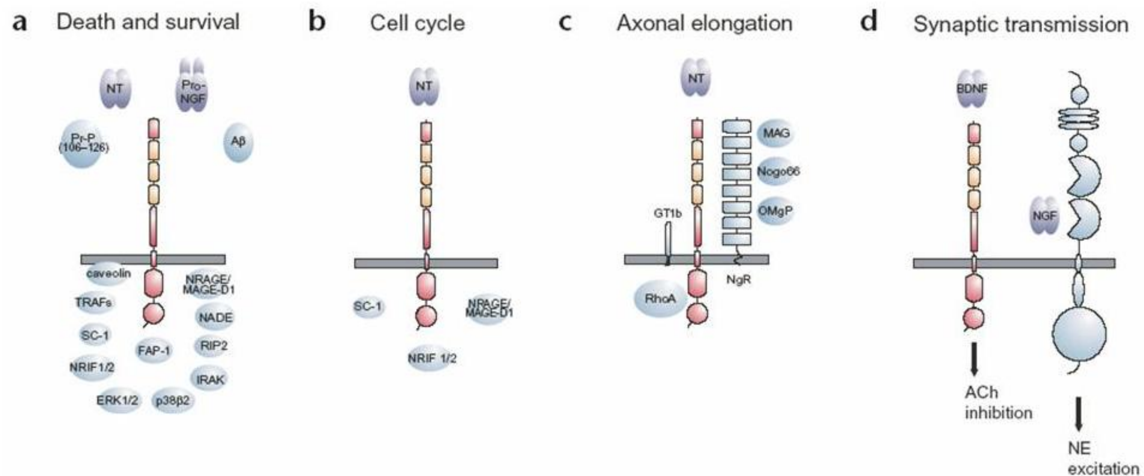
Inoltre, p75^{NTR} è anche coinvolto nella modulazione dell'architettura cellulare, controllando l'attività della fosfatasi Rho [60]. Rho è una piccola guanosin tri-fosfatasi (GTPase) che regola lo stato di polimerizzazione dell'actina. La forma complessata con la guanosin tri-fosfato (GTP) irrigidisce l'actina citoscheletrica impedendone l'ulteriore assemblaggio dei monomeri. In particolare, è stato visto nel processo di elongazione assonale dei neuroni, la forma libera di p75^{NTR} può legare Rho, attivandolo e quindi inibendo l'elongazione assonale, al contrario, il legame di p75^{NTR} con altre NTs, non permette l'attivazione della GTPase a favore del processo di l'elongazione [60]. Inoltre, p75^{NTR} sembra anche coinvolto nel processo di mielinizzazione tramite la Glicoproteina Associata alla Mielina (MAG) [61, 69] e NgR [57].

Per quel che riguarda trasmissione sinaptica e plasticità neuronale, l'NGF sembra mediare il rilascio dell'uno o dell'altro neurotrasmettitore asseconda del recettore che leghi. Neurotrasmettitori differenti sono conservati in vescicole distinte ed i recettori trkA^{NGFR} e p75^{NTR} attivano il rilascio o dell'uno o dell'altra vescicola in maniera indipendente [70, 71]. Precedenti studi indicano che entrambi i recettori dell'NGF possano mediare un aumento di calcio intracellulare [72]. Probabilmente le vescicole contenenti i neurotrasmettitori hanno diverse sensibilità ai diversi livelli di calcio intracellulare [73]. La figura 1.3 mostra una rappresentazione schematica del recettore p75^{NTR} e le possibili interazioni con i suoi co-recettori e ligandi che possono portare a diversi segnali all'interno della cellula.

In ultimo, l'NGF è anche in grado di attivare la cascata delle proteine Smad, un pathway tipico del Trasforming Growth Factor-β1 (TGF-β1). In particolare, nelle PC12, l'NGF sembrerebbe indurre l'associazione Smad3-Smad4, la traslocazione del complesso nel nucleo e la successiva trascrizione genica, in modo specifico [74]. Queste osservazioni hanno portato ad investigare tale pathway mediato dall'NGF, in associazione con il TGF-β1, il fattore chiave

per eccellenza nel rimodellamento tissutale, per lo studio di questo processo[75].

I meccanismi che scaturiscono dal legame dell'NGF alla cellula target sono tuttavia ancora oggi oggetto di vivissimi ed interessanti studi in continua evoluzione.



Dechant & Barde, Nature2002

Figura 1.3: Rappresentazione di una serie di segnali in cui il p75 è coinvolto: (A) p75 regola sia la morte che la sopravvivenza cellulare [76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88]; (B) (C) In coltura, alcune proteine, interagendo con p75, bloccano la progressione del ciclo cellulare. p75 regola anche l'elongazione assonale, sia durante lo sviluppo che dopo una lesione. Il dominio citoplasmatico lega Rho a favore dell'elongazione assonale [60]. L'NgR lega tre differenti inibitori dell'elongazione assonale (mielina associati): la glicoproteina MAG, un frammento della proteina (oligodendrocita associata) Nogo-66 e la glicoproteina mielin-oligodendrocita OmgP. (D) In co-colture di miociti e neuroni simpatici, i singoli neuroni secretano due distinti neurotrasmettitori: l'attivazione di trkA^{NGFR}, via NGF, stimola il rilascio di norepinefrine (NE), e i miociti rispondono con l'aumento della frequenza delle contrazioni, diversamente l'attivazione di p75 tramite BDNF stimola il rilascio di acetilcolina (ACh), con effetto opposto [89]

1.2 L'NGF nel SNC.

Attraverso saggi immunoenzimatici è stato possibile dosare i livelli dell'NGF in varie regioni del SNC di diverse specie di mammiferi. Nella tabella 1.1 sono mostrati i livelli dell'NGF nel

SNC di ratto [90]. Livelli relativamente alti dell'NGF sono presenti in tutte le aree contenenti le terminazioni di neuroni colinergici magnocellulari che dopo internalizzazione e trasporto retrogrado assonale dell'NGF rispondono con un aumento dell'acetilcolintransferasi (AchT) [91, 92]. Tali evidenze sono in accordo con l'ipotesi che l'NGF sia sintetizzato nelle regioni bersaglio dei neuroni magnocellulari colinergici, quindi captato dalle terminazioni di questi neuroni e trasportato in maniera retrograda ai loro corpi cellulari. A sostegno di questa interpretazione è la scoperta che l'RNAm per l'NGF è presente in alte concentrazioni nelle regioni bersaglio dei neuroni colinergici (ippocampo e corteccia), mentre nelle regioni contenenti i corpi cellulari di questi neuroni è scarsamente rilevabile (setto) [93]. Nell'insieme, questi risultati indicano che la presenza dell'NGF nel setto, come negli altri nuclei del telencefalo basale, è dovuta ad un trasporto retrogrado assonale piuttosto che alla sintesi locale [90]. E' interessante notare che il cervelletto, che non riceve fibre colinergiche, presenta livelli dell'NGF relativamente alti e ciò fa supporre un'innervazione di neuroni non colinergici, localizzati nel midollo allungato, responsivi all'NGF [90]. Nel 1990 Aloe e coll. hanno messo in evidenza la presenza dell'RNAm per l'NGF a livello ipotalamico; in quest'ultima struttura cerebrale l'NGF ed il suo messaggero si trovano nel nucleo preottico ed in quello paraventricolare dove la loro sintesi sembra essere regolata da eventi psicosociali stressogeni [94, 95].

Table I. NGF levels in rat central nervous system

Tissue		ng NGF/g wet weight
Hippocampus	Regions innervated by magno-	1.41 ± 0.08
Olfactory bulb	cellular cholinergic neurons[a,b]	0.40 ± 0.04
Neocortex		0.53 ± 0.08
Septum	Regions containing the cell	0.51 ± 0.06
Diagnonal band of Broca	bodies of magnocellular	0.71 ± 0.11
Nucleuc basalis of Meynert	cholinergic neurons[a,b]	0.37 ± 0.04
Corpus striatum	Regions with other types of	0.08 ± 0.01
Retina	cholinergic neurons[a]	0.07 ± 0.01
Hindbrain + Medulla oblongata		0.07 ± 0.02
Hypothalamus		0.16 ± 0.02
Cerebellum	Regions not containing	0.21 ± 0.03
Optic tectum	cholinergic neurons[a]	0.07 ± 0.01

Tabella 1.1: Livelli di NGF nel Sistema Nervoso Centrale. I valori riportati derivano dalle medie (+ SEM) di almeno 4 esperimenti indipendenti ognuno dei quali valutato in quadruplicato [90].

Una volta messa in luce la presenza di NGF e localizzati i siti di sintesi, si è cercato di chiarire l'azione promossa da questa molecola nel SNC. Da diversi studi è emerso che l'NGF gioca un ruolo sui neuroni colinergici del telencefalo basale simili a quello svolto sui neuroni adrenergici periferici. Dal momento che l'AchT e l'acetilcolinesterasi (AchE), gli enzimi implicati rispettivamente nella sintesi e nella degradazione dell'acetilcolina, sono considerati i marcatori ideali dei sistemi colinergici, la loro attività è stata utilizzata per analizzare la natura dell'azione dell'NGF sui neuroni telencefalici basali. I risultati nel complesso indicano che l'NGF esogeno ha la capacità di influenzare i neuroni colinergici con un effetto dose età dipendente. Ulteriori informazioni sul ruolo fisiologico svolto dall'NGF a livello del SNC sono state ottenute mediante l'iniezione in vivo di anticorpi diretti contro l'NGF. Questi studi hanno evidenziato la presenza di un periodo critico durante lo sviluppo in cui i neuroni colinergici sono più sensibili all'effetto trofico e differenziativo dell'NGF [96]. Uno dei

problemi sull'interpretazione dei risultati ottenuti con gli studi di deprivazione dell'NGF in diversi momenti dello svoluppo del SNC è costituito dal fatto che durante gli stadi fetali e nei primi giorni di vita postnatale molti dei sistemi colinergici centrali non si sono ancora sviluppati [97]. Per spiegare questa discordanza temporale tra gli effetti dell'NGF e la differenziazione dei suoi bersagli è stata avanzata l'ipotesi che l'anticorpo somministrato in un periodo di alta sensibilità possa restare localizzato nelle regioni cerebrali specifiche fino all'inizio del differenziamento dei neuroni colinergici [96]. Alcuni ricercatori hanno dimostrato che lesioni della struttura setto-ippocampale e transezioni della fimbria determinavano una forte crescita reattiva di fibre nervose simpatiche periferiche nella regione dell'ippocampo, mentre non si osservavano questi stessi effetti interrompendo le vie afferenti all'ippocampo (vie entorinali e l'imput adrenergico dal locus coeruleus) [98]. Sebbene questi risultati sostenessero la possibilità che la forte crescita di fibre simpatiche nell'ippocampo dopo la lesione, fosse almeno in parte dovuta all'NGF, la prova finale di un'azione trofica di questa molecola nel SNC è stata fornita dall' esperimento di Springer e Loy (1985) [99]. Questi ricercatori dimostrarono che l'iniezione locale di anticorpo anti-NGF nell' ippocampo era in grado di abolire la crescita neuritica simpatica, dopo lesione della fimbria, malgrado l'effetto di tale iniezione fosse limitato alle immediate vicinanze del sito di iniezione a causa della scarsa diffusibilità dell'anticorpo. Inoltre, inaspettativamente, Gasser e coll. nel 1986 trovarono che in seguito alla lesione della fimbria i livelli di NGF non erano diminuiti nella regione del setto, come era ipotizzabile per effetto dell'eliminazione del trasporto retrogrado, bensì aumentati in modo marcato [100]. Questi risultati furono interpretati considerando che l'azione meccanica diretta sul setto o indiretta attraverso la fimbria innesca un processo di gliosi in cui le cellule gliali sono le vere responsabili dell'aumentata produzione dell'NGF e di conseguenza dell' aumentata produzione di AchT nei neuroni colinergici sopravvissuti agli insulti. Inoltre, la continua somministrazione dell'NGF esogeno si dimostrò capace di proteggere il 50% dei neuroni colinergici che altrimenti sarebbero degenerati dopo la lesione della fimbria.

In conclusione questi risultati indicano che l'NGF è un fattore endogeno trofico e differen-

ziativo dei neuroni magnocellulari colinergici del telencefalo basale suggerendo un possibile impiego farmacologico di questa molecola nelle neuropatie degenerative a carico di questa popolazione neuronale.

1.3 Il ruolo dell'NGF nel mediare la "Sensibilizzazione" periferica

Come ampiamente discusso nei precedenti paragrafi, l'NGF è un fattore trofico critico per lo sviluppo e la sopravvivenza dei neuroni del'SNC [4, 101](Levi-Montalcini & Angeletti, 1968; Ritter A.M et al., 1991). Tuttavia, in animali adulti, questo non sembra essere così indispensabile per la sopravvivenza [102](Lindsay, R.M., 1988) quanto invece per il mantenimento dell'omeoastasi dei neuroni, soprattutto per la loro stessa "rigenerazione" a seguito di un danno tissutale [103, 104](Diamond, J et al.,1992a/b). Nel mondo scientifico, è ormai diffusa l'idea che negli adulti l'NGF medi soprattutto le risposte infiammatorie ed immunitarie a seguito di un danno tissutale, soprattutto per dare inizio e mantenere poi "l'ipersensibilità", sintomo di allarme di infiammazione [105, 106](Levi-Montalcini et al., 1996; Pezet S & McMahon SB, 2006). L'ipersensibilità si manifesta come un aumento della sensibilità agli stimoli nocicettivi (iperalgesia) e/o alla percezione di uno stimolo non nocicettivo. Un meccanismo responsabile della ipersensibilità è la ridotta soglia di allarme ad un aumento nell'eccitabilità di piccoli neuroni sensoriali che comunicano informazioni nocicettivi al SNC. Tale fenomeno, terminata la sensibilizzazione periferica, aumenta l'eccitabilità dei piccoli neuroni sensoriali che si traduce in un aumento nel rilascio di trasmettitori dalle loro terminazioni (Figura 1.4). L'aumento di rilascio di questi trasmettitori, come la sostanza P (SP) e la "calcitonin-gene related peptide" (CGRP), contribuisce all'istaurarsi del fenomeno di "infiammazione neurogenica" [107](Richardson JD & Vasko MR, 2002). Il rilascio sostanziale di questi mediatori dalle terminazioni dei neuroni del'SNC può aumentare le funzioni sinaptiche tra le terminazioni

dei nervi sensoriali e i neuroni del midollo spinale [108]. Questo fenomeno, conosciuto come "sensibilizzazione centrale", contribuisce all'iperalgesia e può essere considerato il meccanismo predominante nelle sindromi del dolore cronico.

La capacità dell'NGF di aumentare le risposte nocicettive ha riscosso molta attenzione e ha lanciato l'idea che agendo sull'azione di questa neurotrofina si possano intraprendere nuove strade teraupetiche per il trattamento del dolore [109]. Tuttavia, dal momento che NGF agisce su diversi tipi cellulari, dai neuroni sensoriali alle cellule immunitarie, usare antagonisti che bloccano le risposte nociceptive potrebbe essere più controproducente di quanto si speri.

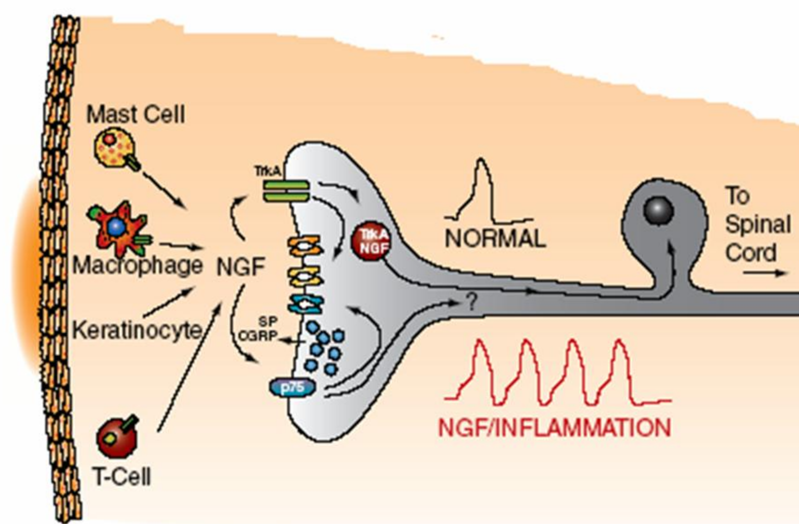


Figura 1.4: Il rilascio di NGF produce sensibilizzazione delle terminazioni dei neuroni sensoriali periferici. A livello di un trauma tissutale, cellule immunitarie come Mastociti, Macrofagi, Cheratinociti e linfociti T rilasciano NGF, che interagendo localmente con i suoi recettori sulle terminazioni nervose. Tale legame attiva una cascata di segnali intracellulari che possono modulare i canali ionici sulla terminazione nervosa o aumentare il rilascio di neuropeptidi : SP e/o CGRP. In normali condizioni, uno stimolo nocicettivo potrebbe produrre un singolo potenziale d'azione, mentre lo stimolo con NGF, o per un trattamento o come conseguenza dell'infiammazione, produrrebbe multipli potenziali d'azione [110].

1.4 NGF e sistema immunitario.

Come altri neuropeptidi anche l'NGF svolge un'attività immunoregolatoria. Tra il 1967 e il 1978 alcuni studiosi hanno evidenziato un ruolo modulatorio delle funzioni immuno-endocrine per l'NGF [21]. A sostegno di tale ipotesi è stato dimostrato che le cellule bersaglio dell'NGF non sono distribuite a caso, ma concentrate in tre aree distinte: nei sistemi nervoso, immunitario ed endocrino. I neuroni adrenergici del sistema simpatico, altamente recettivi a questo peptide, si ritrovano ad innervare organi che mediano la risposta immunitaria come il timo, la milza, i linfonodi, il tessuto linfatico associato all'intestino ed al midollo osseo. Inoltre, non solo i meccanismi neuronali ma anche altri stimoli come l'infiammazione, influenzano la sintesi dell'NGF. Infatti, alte concentrazioni di NGF sono state riscontrate in essudati infiammatori ed in granulomi di specie diverse come il topo, il coniglio, il ratto e la scimmia [4, 111]. Peptidi come le tachichinine, svolgono un ruolo di trasmissione sensitiva nocicettiva, mediano la risposta infiammatoria neurogenica e regolano la risposta immunitaria sembrano modulati dall'NGF [112]. Il trattamento di ratti adulti con l'NGF aumenta la sensibilità verso stimoli nocicettivi, mentre quello con anticorpi anti-NGF riduce sia la risposta al dolore che infiammatoria [113]. Inoltre, l'NGF è in grado di aumentare il numero dei mastociti nei tessuti di ratti neonati [114], ed iniettato per via intradermica, crea uno specifico rilascio di istamina con conseguente aumento della permeabilità vascolare cutanea [115]. L'NGF favorisce anche il processo di cicatrizzazione [115, 111], induce un cambiamento nella forma delle piastrine [116], promuove la differenziazione di basofili, mastociti ed eosinofili [117] ed in assenza di altri agenti aumenta la risposta proliferativa degli splenociti di ratto [118, 119]. L'NGF induce anche la proliferazione dei linfociti B, la differenziazione delle cellule B in plasmacellule e la produzione dei linfociti T [117, 120]. E' stato inoltre osservato che sia cellule linfocitarie [121], che macrofagi attivati, possano utilizzare e rilasciare NGF [122, 123].

Ad oggi è altresì noto che l'NGF risulta essere in grado di influenzare direttamente il sistema immunitario, sia in condizioni fisiologiche che patologiche, rafforzando quindi l'ipotesi

che questo fattore pleiotropico possa svolgere funzioni specifiche nell'interazione tra le diverse cellule immunitarie. La figura 1.5 riporta in modo schematico il contributo dell'NGF al network immunitario e sommarizza i tipi cellulari che ad oggi sono noti essere target dell'NGF.

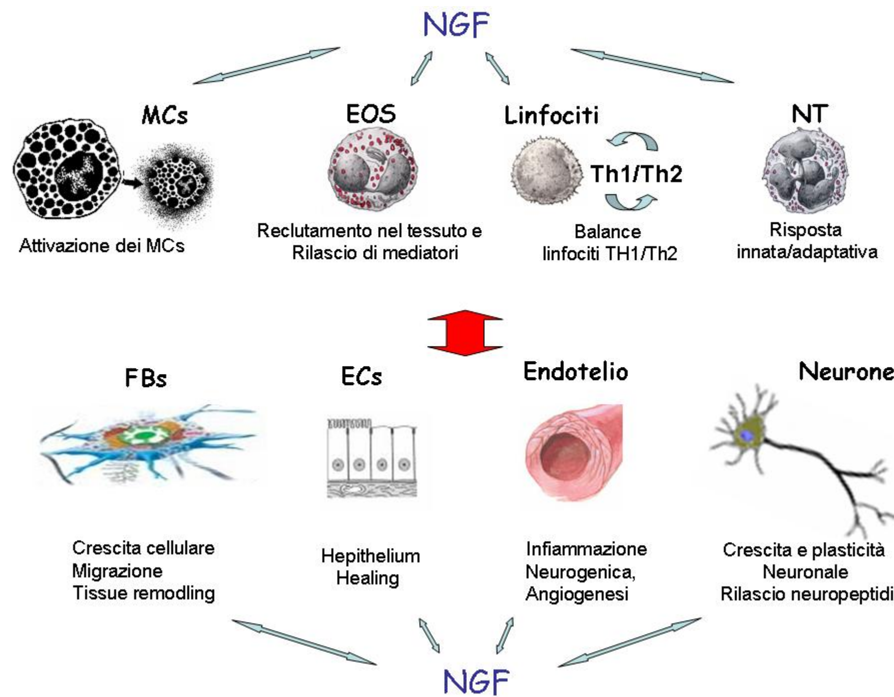


Figura 1.5: Schema riassuntivo del network immunitario e noti target cellulari dell'NGF. Cellule immunitarie. MCs: Mastociti; EOS: eosinofili; Linfociti; NT: neutrofili. Cellule strutturali. FBs: fibroblasti; ECs: epiteliali; Endotelio; Neurone.

1.5 NGF nel sistema visivo e suo coinvolgimento nelle patologie fibrotiche croniche della superficie oculare.

Durante lo sviluppo del sistema visivo, l'NGF ed entrambi i suoi recettori sono espressi in numerose aree visive distribuiti dalla retina alla corteccia visiva [124]. Sempre in questa fase dello sviluppo, l'NGF sembra influenzare la crescita, la sopravvivenza e la morte selettiva neuronale [125, 126, 127]. Esperimenti su topi transgenici, privati di entrambi i recettori,

sono stati utili nel comprendere il ruolo chiave dell'NGF nel trofismo e nell'innervazione della cornea [31, 128]. L'azione dell'NGF non è tuttavia confinata alla sola cornea ma si esplica anche nella fisiopatologia dei disturbi che interessano sia il segmento anteriore che quello posteriore dell'occhio [129, 130, 126]. Relativamente al segmento posteriore oculare, è stato evidenziato in particolare, che iniezioni intraoculari di NGF promuovono il recupero di cellule gangliari retiniche (RGCs), danneggiate a seguito di ischemia o altri traumi. Al contrario, la somministrazione di anticorpi neutralizzanti l'NGF non procura alcun beneficio alle cellule danneggiate [131]. Su diversi tipi cellulari della retina, l'NGF sembra esercitare molti effetti come la proliferazione, la sintesi di mediatori, cambiamenti citoscheletrici, la trasmissione sinaptica, la riorganizzazione e plasticità neuronale [126]. In condizioni fisiologiche, l'NGF è espresso insieme al suo recettore trkA^{NGFR}, sul segmento anteriore dell'occhio (iride, corpo ciliare, lente, cornea e congiuntiva) e quindi rilasciato nell'umor acqueo [132]. Modelli sperimentali hanno permesso di valutare non solo l'abilità da parte della cornea e la congiuntiva di produrre e rilasciare l'NGF ma anche la loro recettività a questa neurotrofina in quanto queste aree esprimono i recettori essenziali per mediare le attività biologiche dell'NGF [133]. Il pathway NGF/trkA^{NGFR} è alterato in condizioni di infiammazioni croniche dell'occhio come nel caso della Cheratocongiuntivite primaverile (o Vernal Kerato Conjunctivitis: VKC) e dell'Ocular Cicatricial Pemphigoid (OCP) [134]. Le funzioni biologiche dell'NGF sono state studiate mediante le osservazioni di casi clinici, e confermate attraverso l'uso di modelli sperimentali che hanno mostrato un aumento dell'NGF probabilmente al fine di promuovere la risoluzione del danno corneale associato alla patologia [135, 136].

I fattori di crescita rivestono un ruolo importante nel mantenimento del film lacrimale e nella regolazione della cornea e congiuntiva, l'NGF è infatti rilasciato nelle lacrime ed insieme ai suoi recettori, risulta essere espresso dal tessuto ghiandolare lacrimale [137, 138, 139]. I vari elementi della superficie oculare (cornea, congiuntiva, ghiandole lacrimali principale ed accessorie) insieme alle interconnessioni nervose costituiscono un'unità morfo-funzionale, pertanto variazioni nel feedback dell'NGF potrebbero portare all'insorgenza di dry eye [138].

Considerando che l'NGF può aumentare la produzione di lacrime, la densità delle cellule caliciformi congiuntivali e le funzioni corneali, esso è stato recentemente utilizzato in un modello animale di dry eye [140].

L'omeostasi della superficie oculare dipende dal mantenimento di un'equilibrata proliferazione, differenziazione, attivazione e sopravvivenza delle cellule epiteliali e stromali [137]. Tale processo avviene sotto il controllo di una complessa rete di fattori di crescita e citochine rilasciati nel film lacrimale dalla ghiandola lacrimale e dalle cellule epiteliali per essere utilizzati dalla cornea e congiuntiva. Oltre a questo pathway esocrino, esiste un network paracrino (epithelium/stroma), che svolge un importante ruolo nell'indirizzare i fattori trofici ed i segnali di allerta ("alert" signals). Nel decorso di patologie infiammatorie e traumatiche della superficie oculare sia l'NGF che il suo recettore specifico trkA^{NGFR} sono over espressi in tutti i compartimenti [132]. In particulare, è stato visto che il rapporto tra NGF e i suoi recettori risulta essere alterato nelle patologie infiammatorie croniche di tipo allergico, che spesso evolvono in un rimodellamento tissutale non fisiologico e che presentano livelli di NGF più alti rispetto alla norma nel siero, nelle lacrime e congiuntiva [141]. Tra le patologie infiammatorie croniche, in cui è implicato l'NGF, un esempio particolarmente studiato è la VKC (patologia mediata da linfociti "T helper"[6] di tipo 2 (Th2)) [142]. Questi pazienti presentano un aumento significativo di NGF circolante accompagnato da un'innalzamento dei livelli di proteine cationiche degli eosinofili (ECP) e IgE totali, due comuni marker periferici di allergia [141]. La stessa correlazione significativa è stata osservata tra i livelli di NGF e gli aumenti nel numero di MCs nella congiuntiva di VKC. Sebbene, Bonini e collaboratori abbiano confermato un possible legame tra l'aumento di NGF e MCs/EOs infltrati [141], non

[6]Conosciute anche come cellule effettrici Th, sono un sottogruppo di linfociti con l'importante ruolo di stabilire e massimizzare le capacità del sistema immunitario. Queste cellule non hanno capacità citotossica o fagocitaria; essi non possono uccidere direttamente cellule o patogeni infettivi, ma hanno bisogno dell'ausilio di altre cellule immunitarie. Le Th sono sono coinvolte nell'attivazione e nel dirigere altre cellule immunitarie, sono essenziali nel determinare l'idiotipo anticorpale liberato dai linfociti B, nell'attivazione e crescita di linfociti T citotossici e nel massimizzare l'attività fagocitaria dei macrofagi contro i batteri. Per la loro diversità funzionale e per il loro ruolo nell'influenzare altre tipi cellulari gli è stato dato il nome di cellule T "helper".

è ancora chiaro quali siano le cellule responsabili di tale aumento e come l'NGF partecipi all'infiammazione allergica. Probabilmente sia i MCs attivati che gli EOs potrebbero essere una fonte importante di NGF, come dimostrato sia in vitro che in vivo [143](Aloe et al., 1999). Inoltre, anche le cellule residenti, come le epiteliali ed i FBs, potrebbero partecipare come fonte ulteriore nella produzione della NT [75]. È noto che l'NGF agisce su tutte le cellule chiave dell'infiammazioni allergiche, come i MCs, gli EOs e i linfociti Th2. In particolare, l'NGF induce la differenziazione, la degranulazione (incluso il rilascio di citochine e fattori di crescita), e la sopravvivenza/apoptosi dei MCs, così come l'attivazione, la sopravvivenza/apoptosi, e il rilascio dei mediatori degli EOs [75]. L'infiammazione cronica evolve, dopo un primo danno tissutale, in un processo di riparazione tissutale orchestrato dall'interazione tra cellule infiammatorie e strutturali [144].

La superficie oculare, per le sue caratteristiche strutturali, funzionali ed immunitarie rappresenta un ottimo modello per lo studio degli effetti dell'NGF nei casi di alterazioni locali tessutali e ancora più nella comunicazione tra le stesse cellule residenti (epiteliali-stromali) con le cellule immunitarie, governanti la risoluzione di un danno.

Capitolo 2

Tissue Remodelling

2.1 "Tissue Remodelling"

Il danno tissutale può verificarsi a seguito di vari stimoli, come infezioni, reazioni autoimmuni, agenti tossici, radiazioni e traumi meccanici. Il processo riparativo che ne segue può evolvere in due modi: rigenerativo, nel quale le cellule danneggiate vengono rimpiazzate da cellule dello stesso tipo, restaurando il fenotipo e la fisiologia precedente al danno, oppure patologico, se anche un solo evento del processo sfugge al controllo del complesso "cross-tollk" tra le cellule immunitarie e strutturali.

Diversamente dalle reazioni infiammatorie acute, un'infiammazione cronica persistente nel tempo può evolvere in fibrosi. Il tentativo da parte delle cellule residenti di risolvere il danno fallisce, perchè continuamente sottoposti a stimoli immunitari che producono fattori di crescita, enzimi proteolitici, fattori angiogenici e citokine fibrogeniche che stimolano la deposizione di elementi del tessuto connettivo che progressivamente rimodellano e deformano la normale architettura del tessuto [145, 146, 147]. Le conseguenze di un cattivo "wound healing" possono essere catastrofiche. In alcune patologie come la fibrosi idiopatica polmonare, cardiovascolare, la cirrosi epatica così come le sclerosi e nefriti sistemiche, soggetti a continui eventi di rimodellamento e fibrosi, possono portare ad un annullamento delle funzionalità

dell'organo fino alla morte del paziente. Quando il tessuto epiteliale e/o endoteliale viene danneggiato, immediatamente le stesse cellule locali rilasciano mediatori infiammatori che innescano una serie di eventi immunitari a cascata, tra cui la produzione degli anti-fibrinolitici che partecipano alla formazione del "clot" del coagulo e la sintesi di una provvisoria matrice extra-cellulare (ECM) [148, 149]. Allo stesso tempo incomincia ad aumentare la vasodilatazione e la permeabilità dei vasi sanguigni, mentre i fibroblasti (FBs), le cellule epiteliali (ECs) ed endoteliali incominciano a produrre metalloproteinase (MMPs) che distruggono la membrana basale, permettendo alle cellule infiammatorie di raggiungere il sito danneggiato. In questo contesto vengono così prodotti una serie di fattori di crescita, citochine, e chemochine che stimolano la proliferazione ed il reclutamento dei leucociti a livello del loco tissutale da ripristinare, attraverso l'ECM provvisorio. I macrofagi e i neutrofili sono tra le prime cellule che si attivano per eliminare i detriti nel tessuto, cellule morte ed organismi invasori. Inoltre, rilasciano citochine e chemochine con l'effetto di richiamare i leucociti ed altre cellule immunitarie e strutturali intorno il sito da riparare, promuovono la formazione di nuovi vasi e la migrazione sia di cellule epiteliali che endoteliali al centro tessuto danneggiato. In questa fase, i linfociti ed altre cellule attivate secernano citochine e fattori pro-fibrotici come TGF-β1, IL-13 e PDGF [150, 145, 151], i quali attivano sia i macrofagi che i FBs. I FBs attivati si trasformano in myo-fibroblasti (myoFBs), i quali differiscono per il loro citoscheletro costituito da α-smooth muscle actin (αSMA), che gli permette di migrare all'interno del sito danneggiato. A seguito di questa attivazione, i myoFBs promuovono la contrazione del tessuto intorno al danno, in cui, i margini si richiudono verso il centro. Alla fine, le cellule epiteliali ed endoteliali si dividono e si dispongono al disopra della membrana basale per rigenerare gli strati superficiali del tessuto completando il processo di "wound-healing". Tuttavia, i tentativi di riparo possono fallire durante casi di infiammazioni croniche. Questi eventi possono portare ad un accumulo eccessivo dei componenti dell'ECM, determinando la formazione di una cicatrice fibrotica permanente, non più funzionale. L'emivita del collagene e dei componenti ECM, regolata dalle MMPs e dai loro inibitori TIMPs, è considerata

una fase chiave nel processo riparativo; infatti, cambiamenti nel loro catabolismo determina l'aumento o il decremento del collagene nel danno [152].

La fibrosi si istaura quando la sintesi di collagene da parte dei myoFBs eccede rispetto a quello che viene degradato, così che il totale ammontare di collagene aumenta sistematicamente col passate del tempo. Proprio per questi motivi i FBs e i fattori che li stimolano, sono i principali target nello studio degli eventi fibrotici.

2.2 Formazione e funzione dei myoFBs durante il "Tissue Repair".

I myoFBs rappresentano le principali cellule che guidano la ricostruzione fisiologica o patologica di un tessuto danneggiato [153, 154]. La differenziazione dei myoFBs può essere schematizzata in un processo a due fasi. Inizialmente, a seguito di cambiamenti nella ECM e della tempesta citochinica nella sede del danno, i FBs acquistano la capacità migratoria, trasformandosi in "proto-myoFBs". Il cambiamento è determinato dalla sintesi di una diversa componente citoscheletrica, le "fibre di stress", che conferiscono ai "proto-myoFBs" una limitata motilità [146, 155, 156]. I "proto-myoFBs" a causa del continuo stress nell'ECM, dovuto al loro stesso remodelling, ultimano il processo differenziativo in "myoFBs". Tale fase è caratterizzata dall'espressione di αSMA, considerato il marker più rappresentativo per la loro identificazione [155]. L'espressione di questa particolare actina è regolata dall'azione combinata di numerosi fattori: specifiche proteine della ECM, come la fibronectina (nella sua variante ED-A), fattori di crescita, come il TGF-β1, e da stress meccanici [146]. L'inserimento di αSMA nelle fibre di stress aumenta significativamente l'attività contrattile dei myoFBs, marcando la fase di contrazione del rimodellamento tissutale connettivale [157].

Nel "Tissue remodelling" fisiologico i myoFBs terminano la loro attività contrattile a riparazione ultimata, quando l'espressione di αSMA decresce e conseguentemente i my-

oFBs muoiono per apoptosi [158]. Nel corrispettivo patologico, non vi è una cessazione dell'attività dei myoFBs da cui si determina una deformazione tissutale come accade nelle cicatrici ipertrofiche da ustione, nella fase fibrotica della sclerodermia, nella sindrome di Dupuytren ed in molti altri casi non qui elencati.

2.2.1 Fattori implicati nella regolazione dei myoFBs

Il TGF-β1 è considerato il principale fattore implicato nella differenziazione dei myoFBs che promuove l'espressione di αSMA [159, 160], la nuova sintesi di proteine dell'ECM [156] e di numerose componenti del citoscheletro [161].

Il pathway atraverso il quale TGF-β1 regola l'espressione di αSMA coinvolge la reazione a catena delle proteine Smad (Figura 2.1 [157]). Il TGF-β1 attivato è riconosciuto da due recettori distinti che vengono reclutati in sequenza per mediarne il segnale. Inizialmente viene legato il recettore di tipo II che, dopo fosforilazione, recluta il recettore di tipo I, formando un complesso eterodimerico. Il complesso possiede un'attività serina/treonina kinasica e fosforila la Smad3 (Smad2, in alcuni casi) che a sua volta lega Smad4 e trasloca nel nucleo. A questo punto avviene l'amplificazione della trascrizione di diversi geni, tra cui quello dell'αSMA, grazie all'interazione del complesso con i fattori di trascrizione del DNA [162, 163]. L'inibizione di questa cascata di eventi è determinata dal sequestro di Smad3 esercitato dal fattore citoplasmatico Smad7.

Anche altri fattori intervengono nella regolazione dell'attività dei myoFBs, di cui alcuni cooperano con TGF-β1 mentre altri ne sono antagonisti. Tra i cofattori solubili, il fattore di crescita del tessuto connettivo (CTGF) è il mediatore meglio studiato, che potenzia l'attività profibrotica del TGF-β1 ed induce la proliferazione dei FBs [164]. La sua attività pleiotropica è da attribuirsi ai suoi sottodomini strutturali e all'interazione mutualmente esclusiva con altre citochine come l'epithelial growth factor (EGF) ed l'insulin growth factor 2 (IGF-2) [165]. Tra le proteine di membrana va annoverata la galectina-3 implicata nella stimolazione

della differenziazione dei myoFBs in coltura di cellule stellate epatiche [166].

Tra i fattori solubili antagonisti, va ricordata l'IL-1, vista inibire in vitro, l'espressione di αSMA, e quindi la differenziazione dei FBs in myoFBs [167]. Inoltre, la sovraespressione di "IL-1 receptor antagonist" intracellulare sembra avere effetti simili [168]. Un'altra citochina, antagonista al TGF-β1, è l'interferonγ (INFγ), rilasciato dalle cellule T (risposta Th1) il quale sembra ridurre l'espressione di αSMA in FBs in coltura. Tale effetto sembra mediato dall'attivazione di una proteina repressore YB-1, che trasloca nel nucleo e interferisce con la trascrizione genica indotta da TGF-β1, interferendo con il segnale di Smad 3 e promuovendo la trascrizione di Smad7 [169, 170].

Alcuni fattori sono stati visti indurre il differenziamento dei myoFBs, indipendentemente dal TGF-β1, almeno apparentemente. Esperimenti condotti su topi transgenici, deleti del gene per L'IL-6, mostrano un alterato "woun healing" cutaneo, ed un ridotto numero di myoFBs, indipendentemente dai livelli di TGF-β1 [171]. Similarmente, in altri studi è stato visto che l'NGF aumenta l'espressione di αSMA, in culture di FBs della pelle e del polmone [172]. Tuttavia, nel 2005 Micera e suoi collaboratori hanno dimostrato in vitro che l'effetto pro-fibrotico dell'NGF su FBs, congiuntivali umani, è dovuto all'effetto endogeno del TGF-β1, stimolato dall'NGF stesso [173].

2.2.2 Attività dei myoFBs regolata dal sistema immunitario innato e selettivo.

Disordini con un'evoluzione di tipo fibrotico presentano spesso un'eziologia infettiva, di tipo batterica, virale o funginea che nel tempo si cronicizza. È stato recentemente visto che patterns molecolari associati ai patogeni (PAMPs), trovati su organismi infettivi, mantengono i myoFBs in uno stato di continua attivazione [174]. Con il nome di PAMPs ci riferiamo a sottoprodotti patogeni come lipoproteine, DNA batterico o RNA a doppio filamento i quali sono riconosciuti da specifici recettori, i PRRs, che sono espressi da un gran numero di cellule

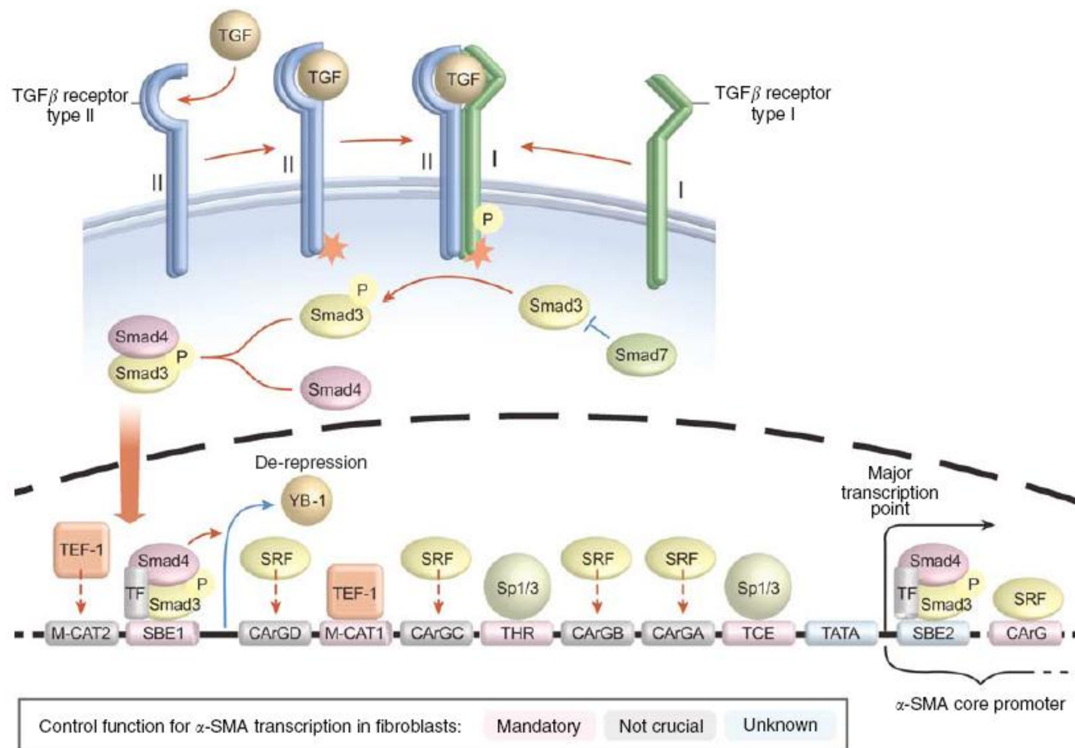


Figura 2.1: Regolazione della trascrizione di αSMA nei myoFBs. L'espresssione di αSMA in myofibroblasts è mediato dall'attivazione del complesso recettoriale del TGFβ1, attraverso il pathway delle Smad, che porta all'associazione di Smad3 a Smad4, che traslocano nel nucleo. Smad3 riconosce e si lega al DNA a livello del promotore per l'αSMA, coniugato ad altri fattori di trascrizione (TF). Fattori antagonisti del TGFβ1, come l'IFNγ, attraverso l'attivazione di YB-1 che inibisce la trascrizione di Smad3 e up-regola l'espressione dell'inibitore Smad7, down-regolano la trascrizione dell'αSMA. TGFβ1 può indurre la trascrizione di αSMA indipendentemente dalle Smad, mediante il riconoscimento e il legame dei kru¨ ppel-like factors Sp1/3 all'elemento di controllo del TGFβ1 (TCE) e alla regione ipersensibile del TGFβ1 (THR). Inoltre, mentre il legame di TEF-1 all'elemento genomico MCAT-1 è cruciale, non lo è l'interazione dei "serum-response factor" (SRF) con le regioni A,B,C e D degli elementi CArG per la trascrizione di αSMA in myoFBs. (figura da: Hinz, 2007[172])

tra cui i FBs [175].

Le iterazioni tra le PAMPs e i loro recettori rappresentano la prima linea di difesa durante le infezioni e attivano numerose citochine e chemochine proinfiammatorie. Gli stessi FBs possiedono una grande varietà di questi recettori, inclusi i Toll-like receptors (TLRs), che mediante il riconoscimento di tali ligandi possono attivare direttamente i FBs e promuovere la loro differenziazione in myoFBs e quindi la sintesi di nuovo collagene [174, 175, 176]. Tutto questo suggerisce che inibire i segnali dei TLRs potrebbe rappresentare un nuovo approccio per il trattamento di queste malattie fibrotiche. Un meccanismo, indipendente dal segnale TGF-β1/SMAD3 [177, 178], sono le infezioni da virus come il citomegalovirus CMV, il quale stimola la produzione di anticorpi e il CTGF, che partecipano entrambi nell'attivazione costitutiva dei myoFBs [179, 180]. Tuttavia, anche fattori non infettivi, possono portare all'attivazione dei FBs come nel caso della sclerosi sistemica in cui i FBs assumono il fenotipo di myoFBs costitutivamente attivati [181, 179], similmente ai normali FBs stimolati con TGF-β1. I risultati di una serie di modelli sperimentali, mostrano che in alcuni casi la fibrosi non sempre è caratterizzata dalla persistenza dell'infiammazione e ciò significa che i meccanismi che regolano la fibrosi, ad un certo stadio, si separano da quelli dell'infiammazione [182, 145]. In altri studi, è stato visto che le cellule T, di tipo CD4+, hanno un ruolo importante nella progressione della fibrosi, infatti in modelli animali è stata dimostrata una forte correlazione tra la patologia fibrotica del fegato con le risposte di cellule Th2/CD4+ (IL-4, IL-5, IL-13 e IL21) [183, 184, 185, 186]. Al contrario, sembra che le citokine rilasciate dalla risposta immunitaria di tipo Th1, come IFNγ e IL-12 abbiano un potente effetto anti-infiammatorio [183, 187]. Ciò ha indotto a pesare che riuscire a deviare le risposte immunitarie di tipo Th2 in favore di quelle di tipo Th1 potesse ristabilire il giusto equilibrio, necessario per un corretto "wound healing". Tuttavia, nonostante siano noti molti fattori e meccanismi che influenzano i FBs, siamo ancora lontani da un approccio terapeutico mirato che impedisca la perdita fisiologica del tessuto colpito, probabilmente perché non una ma la concausa di più fattori, genetici ed ambientali, cooperano nello stabilire e mantenere i myoFBs sempre attivati.

Capitolo 3

Patologie fibrosanti ed NGF: finalità del progetto

Per definizione, l'infiammazione è una reazione locale ad un danno tissutale localizzato, che tenta di risolvere e riparare gli effetti del danno. I fattori che possono dare il via alla risposta infiammatoria sono molteplici ed includono agenti infettivi, fisici, (bruciature, radiazioni, traumi, ecc.), chimici (farmaci, tossine, ecc.) e agenti immunologici come allergeni nel caso di malattie allergiche o autoantigeni nel caso di malattie autoimmuni.

Indipendentemente dalle cause scatenanti, alla risposta infiammatoria acuta partecipano quasi sempre le stesse cellule e fattori solubili e cellulari. Anche quando l'evento infiammatorio si prolunga, come nel caso di persistenza dell'agente causale, la risposta è identica: la lesione non può essere riparata, con l'acqusizione di un fenotipo cicatriziale alterato e perdita della funzionalità dell'organo in questione.

L'NGF è presente in svariate condizioni patologiche sia come mediatore del dolore sia come immunomodulatore (book: Stampachiacchiere et al., 2008; New Immunology Research Developments) cellulare e di altri mediatori dell'infiammazione come ad esempio il TNF-α, TGF-β1, INF-γ o l'IL-1β [188, 173, 189].

3.1 Allergie

L'infiammazione allergica è l'insieme di quelle malattie: asma, rinocongiuntivite allergica, dermatiti, shock anafilattico che sono caratterizzate da sintomi organo specifici e che gli individui predisposti possono sviluppare quando vengono in contatto con un allergene. Il sistema immunitario di questi soggetti, che rappresentano circa il 20% della popolazione occidentale, reagisce velocemente a sostanze esogene di carattere antigenico ma non patogene e quindi chiamate "allergeni" (pollini, profumi, medicine, secrezioni animali e persino alcuni alimenti).

Questa reazione porta all'attivazione di un particolare tipo di linfociti, i Th2, che aiutano i linfociti B a sintetizzare gli anticorpi dell'allergia, le IgE specifiche per l'allergene che le provoca. Le IgE si legano ai recettori specifici ad alta affinità (FcεR1) del mastocita, cellula principe della risposta allergica. I mastociti sono distribuiti in vari tessuti di organi come la pelle, i bronchi, la mucosa nasale ed oculare, l'utero, il cuore, ecc. Quando l'allergene penetra nell'individuo, precedentemente sensibilizzato (che ha già prodotto IgE specifiche), viene riconosciuto dai mastociti innescando il segnale di attivazione cellulare. Si ha quindi la degranulazione di queste cellule con conseguente rilascio di mediatori fra cui l'istamina, la triptasi, la chimasi, citochine come il TNF-α, IL-3, IL-4, IL-5, il "Granulocyte Macrophage Colony Stimulating Factor" (GM-CSF) e fattori di crescita come lo stesso NGF [188]. Questa fase della risposta allergica è chiamata "Fase Precoce", responsabile dei primi sintomi dell'allergia. Alcune ore dopo, si sviluppa quella che è chiamata "Fase Tardiva" in cui cellule infiammatorie (neutrofili, cellule mononucleate: basofili ed eosinofili) si infiltrano nel tessuto. A questo punto è l'eosinofilo (EO) la cellula cardine di questa fase, che permane per tutto il tempo della reazione anche quando diventa cronica. l'EO attivato degranula rilasciando vari mediatori a carattere basico, responsabili del danno tissutale, a volte irreversibile, riscontrabile in quei casi dove l'allergia diventa cronica.

Nel caso che la reazione allergica sia limitata è dimostrabile un'ultima fase di cicatriz-

zazione in cui sia i mastociti che gli eosinofili sembrerebbero implicati come fonte di fattori fibrogenici e quindi riparatori [188].

Vecchi studi, condotti su ratti e topi, hanno dimostrato che la somministrazione di NGF portava alla proliferazione e all'iperplasia dei mastociti in diversi siti anatomici [190], suggerendo un ruolo differenziativo e di crescita per le cellule.

In realtà, l'NGF influenza anche la maturazione ed il fenotipo dei mastociti [143, 142]. Inoltre è stato visto che l'NGF non si limita a regolare la differenziazione, maturazione e sopravvivenza di queste cellule ma anche la loro funzionalità, infatti sembra che l'NGF stimoli la produzione di istamina [191] tramite l'interazione con il suo recettore specifico trkA^{NGFR}, mentre non sembrerebbe espresso dai mastociti il p75^{NTR}. Studi su altre cellule dell'infiammazione hanno rilevato che gli EOs umani esprimono il recettore trkA^{NGFR} e sono indotti dall'NGF a rilasciare EPO [133]. E' stata dimostrata anche la capacità dell'NGF di stimolare colonie granulocitarie ottenute da sangue periferico per indurre la differenziazione selettiva di EOs, basofili e mastociti [188]. Inoltre, l'NGF è anche coinvolto nella chemiotassi, sopravvivenza e attività citotossica dei neutrofili, altre cellule infiammatorie presenti nelle prime ore della risposta allergica [188]. L'NGF potrebbe influenzare la fase riparativa dell'infiammazione, in diversi studi infatti, dove l'NGF è stato topicamente somministrato a pazienti che presentavano ulcere della pelle o delle mucose dell'occhio si è osservata la completa guarigione delle lesioni [135].

La prima correlazione tra alti livelli di NGF e patologie allergiche come la rincongiuntivite, l'orticaria-angioedema e l'asma è stata descritta da Bonini e collaboratori nel 1996[192]. Inoltre, tali livelli rispecchiavano il tasso di IgE e la severità clinica dalla malattia. Lo stesso gruppo ha studiato in particolare una patologia dell'occhio a carattere allergico, la VKC, in cui hanno visto che sia le cellule epiteliali basali, endoteliali che i FBs possiedono recettori per l'NGF [141, 142]. In un modello murino di asma è stato dimostrato che l'infiammazione allergica delle vie aeree è acompagnata da un'elevata produzione locale di NGF, che amplifica le funzioni effettrici delle cellule Th2 e che ha un ruolo importante nell'iperattività

bronchiale [193]. Da allora sono stati fatti molti studi sull'argomento e gli stessi ed altri autori hanno pubblicato diversi lavori a sostegno di un ruolo pro-infiammatorio per l'NGF nell'infiammazione allergica [194, 195]. Dall'altra parte ci sono anche diversi lavori che mostrano un ruolo anti-infiammatorio dell'NGF [196, 197, 198, 199, 189, 200]. Noi in particolare, abbiamo visto che la somministrazione di NGF esogeno e/o anticorpi anti-NGF a ratti trattati con ovalbumina[1] e di controllo, non modificava i livelli di IgE e di EOs nel lavaggio fluido bronchiale alveolare (BALF) [200]. Di conseguenza a questo lavoro, abbiamo supposto che l'NGF agisca come neuro-immune modulatore dello stato allergico regolando i rapporti fra mastociti e cellule nervose, servendo da ponte fra sistema immunitario e nervoso.

3.1.1 Cheratocongiuntivite primaverile: patologia a carattere infiammatorio di tipo allergica

La VKC è una patologia infiammatoria cronica della congiuntiva, caratterizzata da un fenotipo di tipo allergico. Le cellule infiammatorie coinvolte nell'immunità cellulare della patogenesi sono linfociti (principalmente di tipo Th2), EOs, e mastociti (Bonini et al., 2003). Nella VKC l'infiammazione allergica (th2) è associata ad un ri-modellamento tissutale per la comparsa di FBs attivati, crescita epiteliale, fibrosi subepiteliale e deposizione di ECM con conseguente formazione delle papilli giganti [201].

L'analisi dell'ECM, nella congiuntiva di pazienti affetti da VKC, ha mostrato sia un aumento che un'alterazione nei rapporti tra i diversi tipi di collagene (tipo I, III e V) [202]. Questa alterazione è dovuta ad un aumento di espressione di citochine e fattori di crescita dai linfociti Th2 e altre cellule infiammatorie, le quali stimolano le cellule residenti (FBs attivati) nella iper-produzione dell'ECM[202], risultato di uno sbilanciato rapporto tra la produzione

[1] Glicoproteina usata per indurre la reazione allergica su modelli sperimentali. Esistono due varianti genetiche: OvA e OvB, che possono essere distinte con l'elettroforesi. La molecola di ovalbumina è formata da un oligosaccaride unito alla catena polipeptidica. La differenza tra i due tipi risiede nel fatto che OvA possiede un residuo di asparagina al posto di acido aspartico presente in OvB. Questa differenza è causata da una mutazione che si è verificata in corrispondenza del residuo 998 della sequenza nucleotidica

di collagene e MMPs attive della matrice (MMP1, MMP2 e MMP9) [202].

Nella VKC molte citochine, prodotte sia dalle cellule infiammatorie che strutturali, come il TNF-α, IL-1, IL-4, IL-13, IL-6 e fatori di crescita come il TGF-β1, che causano l'insataurarsi dell'infiammazione cronica e cambiamenti strutturali fibrotici, sono state trovate aumentare sia a livello circolatorio, nelle lacrime e nella congiuntiva di questi pazienti [201]. Il TGF-β1 rappresenta il principale fattore profibrogenico, responsabile dello sbilanciamento del metabolismo dell'ECM indispensabile per la sopravvivenza e come fattore chemoattrattore dei FBs/myoFBs. Tra i fattori di crescita, anche l'NGF sembra partecipare nell'interazione tra cellule epiteliali e stromali durante il wound-healing e il processo di riparo [75, 173].

Il fatto che l'NGF aumenti sia nel sangue che nella congiuntiva di questi pazienti, sommato al suo effetto profibrogenico su colture primarie di FBs congiuntivali sani ha portato ad ipotizzare che questa NT possa essere coinvolta nel riparo tissutale di VKC.

3.2 Patologie Autoimmunitarie

E' noto che alterati meccanismi di regolazione della risposta immunitaria compromettono il normale funzionamento dell'organismo che possono portare a lesioni tessutali. Nelle patologie autoimmunitarie questa regolazione è compromessa a tal punto che l'organismo non riconosce alcuni dei sui stessi antigeni endogeni, inducendo la sintesi di auto-anticorpi. In alcune di queste patologie è stato rilavato un aumento di NGF nel siero e nei tessuti lesi di questi pazienti [203, 204, 196, 205]. Probbabilmente, nella maggior parte dei casi, responsabili di questo accumulo sono proprio le cellule infiammatorie che infiltrano nel tessuto. Inoltre, numerosi studi in vitro dimostrano come alcune citochine che regolano il processo infiammatorio (IL-β1, TNF-α, IL-6) inducano la sintesi di NGF in cellule non immunitarie come FBs, cherattinociti e cellule della glia [206]. Poiché l'NGF è prodotto nel sito di infiammazione si è pensato che questa proteina potesse specificatamente favorire tale risposta dei tessuti. In realtà, i meccanismi che ne stimolano la sintesi e il modo in cui questa NT intervenga nella

modulazione del processo infiammatorio sono tuttora oggetto di molteplici studi. Una decina d'anni fa, Rita Levi-Montalcini ipotizzava un ruolo di "molecola sentinella" per l'NGF. In condizioni fisiologiche l'NGF determina un'attivazione delle cellule immunitarie che si preparano a rispondere più tempestivamente alle aggressioni provenienti all'esterno.

Lo studio dei livelli di NGF nel liquido cerebrospinale di pazienti affetti da sclerosi multipla ha evidenziato che durante la fase acuta della malattia è presente un elevata concentrazione di NGF, che diminuisce poi nella fase di remissione [207]. La sclerosi multipla è una patologia neurodegenerativa del sistema nervoso centrale su base autoimmune, caratterizzata dalla distruzione degli oligodendrociti[2] Nei modelli animali è stato visto che l'aumento locale di NGF riduce la degenerazione di queste cellule e quindi il danno, promuovendone la proliferazione, differenziazione e sopravvivenza [208]. E' possibile quindi che l'aumento di NGF nel sito d'infiammazione non sia importante solo per la regolazione della risposta immunitaria ma possa avere un ruolo protettivo nei confronti di alcuni tipi cellulari. A conferma di ciò, è stato mostrato *in vivo* il ruolo modulatorio dell'NGF sull'espressione del complesso di immuno-istocompatibilità[3] (MHC-I/II), da parte dei neuroni motori [189], rafforzzando l'effetto prottettivo per questa NT.

Il fatto che si ritrovi un accumulo di NGF nei siti lesi di pazienti affetti da patologie a carat-

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[2]Cellule che formano il rivestimento protettivo dell'assone e consentono la trasmissione ottimale dell'impulso lungo le fibre nervose.

[3]Il Major Histocompatibility Complex (MHC) è un gruppo di geni polimorfici costituito da 30 unità, localizzato sul cromosoma 6 (nel topo sul cromosoma 17). Le più conosciute codificano per proteine espresse sulla membrana cellulare le quali espletano una funzione di riconoscimento di alcuni agenti proteici da parte dei linfociti T, ma contiene i geni anche di altri importanti peptidi come la 21 idrossilasi, le frazioni del complemento C4B,C4A,BF e C2, la proteina chaperone HSP70 (Heat Shock Proteins. Cioè proteine indotte da danno termico) e i geni della famiglia del TNF. I geni "tipici" del complesso MHC legano frammenti di antigeni ad una porzione di tale molecola rendendoli visibili ai recettori dei linfociti T. Identificati inizialmente perché responsabili del fenomeno del rigetto dei trapianti, si è in seguito potuto verificare l'esistenza di due principali classi di queste molecole (e di questi geni), definiti appunto come Classe I (MHC-I ce ne sono 3 copie per ogni cromosoma) e Classe II (MHC-II ha 6 copie per ogni cromosoma), responsabili di situazioni fisiologiche, e talvolta patologiche, nettamente differenti nell'ambito dell'organismo.

tere auto-immunitario come la sclerodermia sistemica[4] [209], lupus eritematoso sistemico[5] [210], e nel tessuto di pazienti affetti da Miastenia gravis[6][205] e nel fluido cerebrospinale di pazienti affetti da sclerosi multipla [211], ha portato molti ricercatori ad indagare quale sia il ruolo dell'NGF nel decorso sia delle patologie immunitarie che allergiche, precedentemente discusse.

3.2.1 Pemfigoide Oculare Cicatriziale: malattia fibrotica propriamente detta.

Con il termine penfigoide si indicano quelle patologie vescicolo bollose, in cui la mucosa orale è l'area del corpo maggiormente interessata dalle lesioni. Tuttavia, è una patologia sistemica che può colpire altre mucose del corpo. Il coinvolgimento della congiuntiva nel penfigoide delle membrane mucose è chiamato: penfigoide cicatriziale oculale (OCP).

La congiuntiva è una delicata mucosa che copre la superficie sottostante la palpebra e la sclera anteriore, confina con l'epitelio corneale a livello dello strato del "limbus"[7]. Sottostante l'epitelio congiuntivele si trova la sostanza propria (stroma). L'epitelio è costituito approssimativamente da cinque strati cellulari non cheratinizzati, mentre lo stroma sottostante è composto da tessuto connettivo, vasi con linfociti, mastociti, e cellule di Langerhan's.[8][212, 213, 214]. L'epitelio congiuntivale e il sottostante stroma sono connessi da una membrana basale epiteliale (MB) delicata ma fisiologicamente e metabolicamente at-

[4]Malattia cronica ed evolutiva, caratterizzata dall'indurimento e l'ispessimento (fibrosi) della cute in zone più o meno estese della superficie corporea. La malattia colpisce spesso anche i visceri, soprattutto l'apparato gastrointestinale, i polmoni, i reni e il cuore. Queste localizzazioni, in particolare, silenti per mesi o anni, fanno della SSc una malattia grave, invalidante, a volte fatale.

[5]Malattia cronica rara di natura autoimmune, che può colpire diversi organi e tessuti del corpo. Il semplice termine di Lupus si riferisce al Lupus eritematoso sistemico, nonostante esistano altre patologie che comprendono lo stesso nome (per esempio Lupus anticoagulante o sindrome da antifosfolipidi).

[6]Malattia neuromuscolare caratterizzata da debolezza muscolare fluttuante e affaticabilità, causata da anticorpi circolanti che bloccano i recettori colinergici postsinaptici o le proteine MuSK (muscle-specific tyrosine kinases) della giunzione neuromuscolare, inibendo l'effetto stimolante dell'acetilcolina.

[7]Strato cellulare di cellule germinali pluripotenti dell'epitelio corneale

[8]Cellule dendritiche abbondanti nell'epidermide negli epiteli pavimentosi. Il nome deriva dal tedesco Paul Langerhans che per primo le descrisse nella pelle.

tiva (Figura 3.1). Gli autoanticorpi che hanno come target degli elementi molecolari della MB, esercitano un ruolo importante nella separazione dell'epitelio basale dalla sottostante membrana (basale) nella congiuntiva di pazienti con OCP. Pertanto l'OCP è una malattia autoimmune, in cui soggetti geneticamente predisposti, a causa di un insulto iniziale di natura sconosciuta, perdono la tolleranza al self per una componente della MB, la β4-integrina [215] che viene attaccata da auto-anticorpi specifici. L'anomalo riconoscimento del self determina l'attivazione del complemento che porta ad un aumento degli infiltrati infiammatori nel tessuto [214]. Il processo si manifesta con un distacco della membrana basale subepiteliale, con effetti tossici diretti. Le cellule infiammatorie rilasciano citochine e fattori profibrotici come TGF-β1, platelet derived growth factor (PDGF) e fibroblast growth factor (FGF), al fine di promuovere la riparazione tissutale, che però esita nella formazione di cicatrici. L'evento fibrotico subepiteliale è favorito dall'aumentata deposizione di ECM, soprattutto collagene, nella lamina propria congiuntivale [216, 217], maggiormente da parte dei FBs [218, 219]. Analogamente ad altre patologie autoimmuni, l'OCP presenta un'associazione con fenotipi HLA specifici, in particolare all'allele HLA-DQβ1*0301 [220]. Esistono poi delle ipotesi sul secondo fattore ambientale che innescherebbe il pemfigoide nei soggetti predisposti: nella forma idiopatica avrebbero un ruolo alcuni microrganismi mentre nella forma da farmaci sarebbero responsabili alcune sostanze chimiche. Il 25% dei pazienti affetti da fibrosi grave vanno in contro a cecità. La perdita della vista è dovuta al processo di cheratinizzazione dell'epitelio congiuntivale e corneale che accompagna la fibrosi, finendo per compromettere in maniera definitiva la funzione corneale. Attualmente le terapie prevedono l'utilizzo di farmaci ad effetto anti-infiammatorio, come i corticosteroidi, ed immunosoppressivo, come la ciclosporina. E' quindi importante orientare la ricerca verso lo studio dei fenomeni fibrotici per poter intervenire non solo bloccando la risposta immunitaria ma sul processo di "healing", evitando formazioni cicatriziali e metaplasie dell'epitelio.

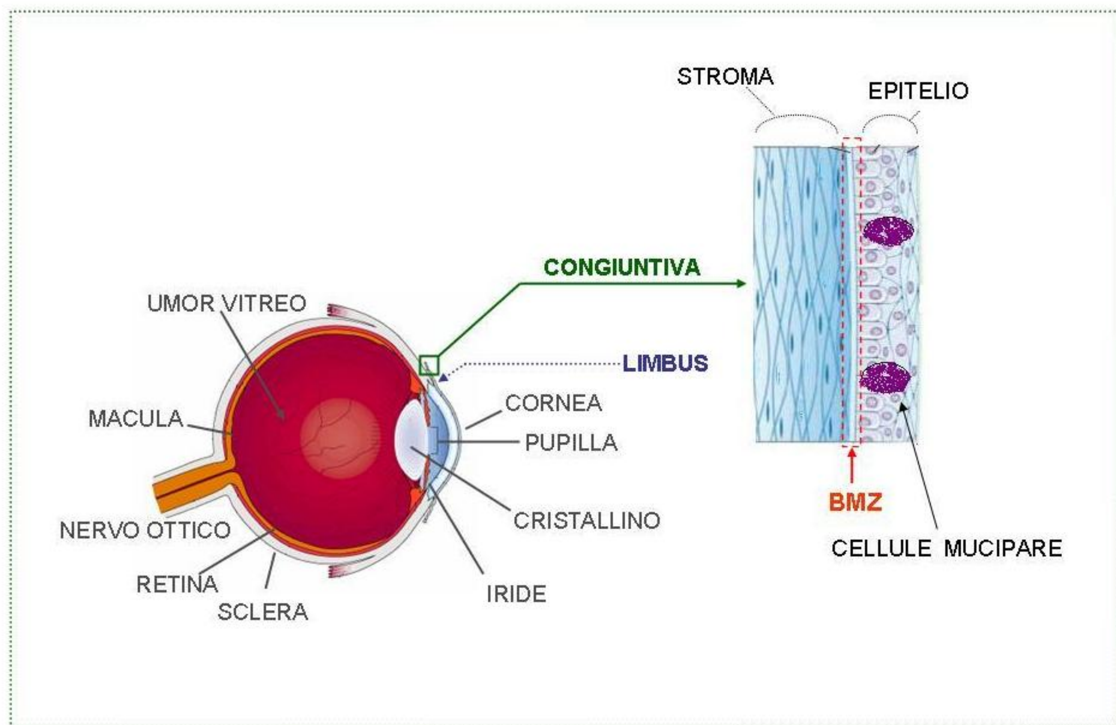

Figura 3.1: Rappresentazione grafica in sezione della superficie oculare. In dettaglio la struttura congiuntivele: dallo strato epiteliale superficiale non cheratinizzato fino alle cellule basali cuboidali con cellule mucipare intercalate, membrana basale (BMZ), e stroma.

3.3 Finalità del progetto:

Grazie a dati ottenuti da vecchi e i più recenti lavori, è stato possibile affermare che l'NGF eserciti un ruolo modulatorio importante nell'infiammazione cronica e nelle diverse fasi fibrotiche che precedono il riparo tissutale, nelle patologie allergiche.

Al fine di comprendere tematiche molto complesse quali sono i meccanismi dell'infiammazione e del rimodellamento tissutale propriamente detto, con questo programma di PhD ci siamo prefisati di studiare e confrontare il coinvolgimento dell'NGF in patologie fibrotiche oculari come la VKC e l'OCP.

I principali punti trattati riguardano:

1. Effetti dell'NGF sul fenotipo FBs/myoFBs di VKC e OCP;
2. Caratterizzazione cellulare di VKC e OCP;
3. Rapporto tra NGF e fenotipo FBs/myoFBs;
4. Possibile modulazione del NGF, in relazione al TGF-β1 ed altre citochine infiammatorie che controllano il delicato equilibrio della risposta Th1/Th2 e dell'ECM;
5. Potenziale contributo dell'NGF nell'immunità innata (TLRs);

Per rispondere a tali quesiti abbiamo proceduto come segue:

Capitolo 4

Materiali e Metodi

4.1 Trattamento dei tessuti, campionamento e procedure sperimentali

Questi studi sono stati approvati dal comitato interno della facoltà di medicina dell'Università Campus Bio-Medico di Roma. Sono state seguite le linee guida della dichiarazione di Helsinki riguardanti la ricerca che coinvolge soggetti umani. I pazienti stessi hanno dato il loro consenso informato per iscritto.

4.1.1 Pazienti, biopsie congiuntivali e colture di FBs

Per VKC: i campioni bioptici congiuntivali sono stati prelevati da 3 pazienti affetti da VKC in forma attiva al momento del prelievo (3 maschi, dagl'11 ai 15 anni). La diagnosi di attiva VKC si basa sulla storia e gli esami clinici, confermati dalla presenza di EOs nelle biopsie. La VKC viene classificata secondo la presenza dei seguenti sintomi oculari: prurito, iper-lacrimazione, fotofobia e sensazione di corpo estraneo; e ciascun segno clinico: iperemia congiuntivale, iper-secrezione di muco, erosione dell'epitelio corneale e delle papille. I campioni di controllo furono sellezionati da bambini e adolescienti (3 maschi tra gli 11 e i 15 anni) che avevano

subito un intervento per strabismo.

Per OCP: i campioni bioptici congiuntivali sono stati prelevati da 7 pazienti affetti da OCP, diagnosticato al momento del campionamento, 2 maschi e 5 femmine, di età compresa tra i 55 e gli 88 anni. La diagnosi di OCP si basa sull'anamnesi clinica del paziente, supportata dall'analisi di laboratorio che consiste in una immunofluorescenza diretta su sezioni del campione bioptico congiuntivele, ottenuto chirurgicamente, che evidenzia la presenza di IgGAM depositate lungo la MB, che può anche essere accompagnata da una positività della marcatura a livello della sostanza intercementante, in alcune zone dello stesso epitelio. Metà delle biopsie sono state quindi incluse in paraffina e poi tagliate in sezioni di 5µm per la diagnosi e per la ricerca, mentre il resto delle biopsie venivano utilizzate per l'ottenimento di colture primarie di FBs congiuntivali.

Diversamente, i campioni congiuntivali usati come controllo sono stati prelevati chirurgicamente da 6 soggetti sani, 5 maschi e una femmina di età compresa tra i 59 e 81 anni, durante l'intervento di cataratta. Anche in questo caso, metà delle biopsie sono state incluse in paraffina e poi tagliate in sezioni di 5µm ma non a fini diagnostici, mentre il resto delle biopsie venivano utilizzate per l'ottenimento di colture primarie di FBs congiuntivali di controllo.

In accordo con i metodi normalmente usati negli studi in vitro, le biopsie di VKC, OCP e quelle di controllo sono state subito piastrate, al momento dell'espianto. In condizioni di sterilità, le diverse biopsie sono state quindi frammentate e poste in piastre da 24 pozzetti, e dopo aver aspettato 10 minuti circa che aderissero alla superficie del pozzetto è stato aggiunto il terreno di coltura: DMEM (terreno arricchito con 10% di Siero Fetale Bovino (FBS) precedentemente inattivato con il calore, glutammina 2mM, antibiotici pennicellina 100U/mL e streptomicina 100μg/mL). Le biopsie venivano lasciate ad incubare a 37°C, in un termostato con aria supplementata al 5% di CO2. Aspettato qualche giorno, che le cellule uscissero dal campione bioptico, e raggiunto una densità cellulare sufficiente, i FBs sono stati tripsinizzati (tripsina 0.2%), lavati 2 volte e subito piastrati in una fiaschetta T-21cm2 per

essere espanse. Per gli esperimenti sono state usate cellule confluenti, dalla terza alla quinta generazione. Nel caso dei FBs di OCP non sono state usate cellule oltre la terza generazione dal momento che i FBs patologici presentavano una morfologia alterata e una scarsa crescita.

La purezza dei FBs congiuntivali è stata testata per esclusione, mediante immunoistochimica del marcatore epiteliale congiuntivale K19 (anticorpo anti-cheratina 19, diluito 1 a 100 della DAKO, Germania). La plasticherai utilizzata per le colture sono forniture NUNC (Roskilde, Denmark), SIAL (Roma, Italia), Celbio (Milano, Italia) e Invitrogen-GIBCO (Carlsbad, CA, USA).

4.1.2 Stimolazione dei FBs a diversi dosaggi di NGF o TGF-β1

Prima di trattare le cellule, cresciute a confluenza in piastre da 6 pozzetti, il monolayers di FBs congiuntivali sono stati mantenuti per 24 ore in terreno povero di FBS (0,5%) per stimolarne la sincronizzazione cellulare e per ridurre l'autofosforilazione [221].

Sia i FBs congiuntivali patologici che quelli ottenuti da interventi di cataratta o strabismo, dopo 24 ore di "starvation", sono stati stimolati con NGF o con TGF-β1. Il β-NGF murino (NF-16), utilizzato negli esperimenti, è stato acquistato da Alomone Lab., Ltd, Gerusalemme, Israele. WEB site: www.alomone.com. Le cellule sono state trattate con concentazioni crescenti di β-NGF: 0,1, 1, 10, 50, 100, 250, 500ng/mL e in parallelo, come controllo profibrogenico, altri FBs sono stati trattati con 1, 5,10,15 e 25ng/mL di TGF-β1 umano (R&D system, Minneapoli, MN) a parità di condizioni. Tutti i FBs, trattati e non, sono stati quindi incubati a 37°C, in ambiente umido: 95% aria e 5% di CO2.

Al termine del trattamento, una parte delle cellule sono state processate enzimaticamente per l'ottenimento o dell'RNA totale o di proteine, usati per le valutazioni molecolari e immunoenzimatiche. In un'altra serie degli stessi trattamenti, le cellule non sono state enzimaticamente distrutte ma fissate per la valutazione delle singole cellule o al confocale o al FACS.

4.1.3 Esperimenti di neutralizzazione

Una procedura comune che permette di studiare gli effetti di una particolare proteina, prevede la sua stessa neutralizzazione. Abbiamo quindi cercato di ridurre l'attività dei recettori trkA^{NGFR}, p75^{NTR} e dei fattori TGF-β1 ed NGF trattando i FBs in studio con anticorpi diretti contro gli stessi. In breve, le cellule confluenti sono state incubate o con 100ng/mL di anticorpo anti-trkA^{NGFR} (Calbiochem, La Jolla, Ca) o con 100ng/mL di anti-p75^{NTR} (Calbiochem, La Jolla, Ca) o con 500ng/mL di anti-NGF (R&D System, Minneapolis, MN) o con 250ng/mL di anti-pan TGF-β1, per 1 ora a 37°C prima di essere trattate con NGF o TGF-β da soli o in combinazione, per 2/6 giorni, secondo le procedure standard (vedi paragrafo 5.1.2).

4.1.4 Test di proliferazione cellulare

Prima di seguire la proliferazione cellulare abbiamo testato la vitalità delle cellule in studio mediante colorazione con Tripan blu. Il primo test di proliferazione eseguito è stato quello della marcatura, secondo le procedure standard ABC, con anticorpi specifici per Ki67 (Tabella 4.1), fattore nucleare di proliferazione espresso durante tutto il ciclo cellulare tranne la fase G0 [222].

Marcatura con timidina marcata H^3: colture cellulari confluenti e subconfluenti sono state incubate con timidina H^3(1μCi/mL) senza e con NGF (0-500ng/mL) o TGF-β1 (10 e 50ng/mL) per un giorno. Le cellule vengono quindi lisate, lavate, precipitate con acido tricloro acetico al 5%, nuovamente lavate con EtOH, dissolte in NaOH 1N e in fine aggiunto il liquido di scintillazione. La radioattività è stata valutata mediante la conta di cellule marcate, in un “beta-counter” (JKB 1211; Rackbeta).

4.1.5 Test di migrazione

La migrazione cellulare verso un danno viene monitorata usando un modello in vitro nel quale viene provocato un taglio lineare al centro di un monolayer di FBs, con una lama di rasoio. In metà del monolayer le cellule vengono raschiate via [223]. Dopo il taglio e rimosso il sovranatante, viene aggiunto il "medium" di coltura, da solo o arricchito con NGF e/o TGF-β1 alle diverse concentrazioni. Le cellule che migrano oltre la linea del tagio vengono quindi monitorate, con un microscopio ottico inverso: Eclipse E400 (5x, Nikon), dopo 2, 4, 12 e 24 ore, contando il numero di griglie che i FBs hanno percorso, dalla linea del taglio al punto più lontano da loro raggiunto.

4.1.6 gel collagene 3D

Colture di FBs in matrici 3D, ricche di collagene, eseguite secondo la tecnica descritta da Tomasek, sono stete utili per valutare la contrazione del gel esercitata dai FBs-cheratociti in studio [224, 225]. Circa 4,5x10^4 /mL di FBs-cheratociti vengono coltivati su dischi da 35mm utilizzati in batteriologia da 35mm, in "medium" al 0,5% FBS e 100nM di NaOH, subito dopo aver aggiunto il collagene di tipo I, ricavato dal tendine della coda di ratto, ricostituito alla concentrazione finale di 2μg/mL, in 18mM di acido acetico in medium da solo o in combinazione con NGF (50, 100, 250, 500ng/mL) o con 10ng/mL di TGF-β1 (pH finale del gel 7.2) [224]. Il diametro del gel viene costantemente controllato giornalmente fino al raggiungimento della massima contrazione, che normalmente avviene entro la seconda settimana. La stima viene eseguita posizionando il disco su un regolo graduato sopra una superficie nera.

4.2 Analisi Biochimiche

4.2.1 Colorazione Ematossilina-Eosina (E&E)

Le sezioni incluse in paraffina sono state trattate per la sparaffinizzazione: il vetrino è messo in stufa a 60°C fino a quando la paraffina non è diventata liquida, incubato per 10 min in xilene e di seguito reidratato.

I vetrini sono stati colorati con E&E secondo la procedura standard:

1. immergere i vetrini 20 sec nel colorante Ematossilina
2. lavare in acqua distillata 2 volte
3. mettere la vaschetta con i vetrini sotto l'acqua corrente di rubinetto per 5 min, possibilmente senza dirigere il getto diretamente sulle sezioni
4. lavare in acqua distillata
5. immergere i vetrini nell'eosina per 1 minuto
6. lavare i vetrini per 2 volte.

4.2.2 Immunofluorescenza (Confocale).

Sia i FBs di OCP che di cataratta, sono stati fatti crescere fino al raggiungimento della confluenza su vetrini coprioggetto (Mierfield, USA), in piastre da 24 pozzetti, e poi preparati e processati per l'analisi al confocale, insieme alle sezioni di tessuto dei rispettivi pazienti. Prima di procedere con l'immunofluorescenza, i campioni tessutali sono stati lavati con un buffer fosfato, preparato in fisiologica, (PBS 1x), mentre le cellule sono state lavate con una soluzione salina di sodio bilanciata: "Hank's Balanced Sodium Salt" (HBSS)). Dopo il lavaggio, tutti i campioni sono stati fissati con p-formaldeide al 2% (PFA)-100mM, preparato in un buffer fosfato (PB). I campioni sono stati quindi permeabilizzati per 10 minuti con

Tabella 4.1: Elenco di anticorpi specifici primari utilizzati per valutare il pathway NGF ed αSMA

Anticorpo	Origine	Diluizione	Casa di Produzione
Anti-hu NGF	Mouse	100	R&D, USA
Anti-hu trkA^{NGFR}	Mouse	60	R&D, USA
Anti-hu trkA^{NGFR}	Goat	60	S. Cruz Biotech, CA, USA
Anti-hu trkA^{NGFR}	Rabbit	60	S. Cruz Biotech, CA, USA
Anti-hu p75^{NTR}	Goat	75	S. Cruz Biotech, CA, USA
Anti-hu αSMA	Mouse	60	Novocastra, Italia
Anti-hu IgGAM	Goat	100	Biotechnology
TLR-4	Rabbit	100	S. Cruz Biotech, CA, USA
TLR-9	Goat	100	S. Cruz Biotech, CA, USA
Ki67	Rabbit	1000	S. Cruz Biotech, CA, USA

0.5% di Triton X-100 in PBS (TX-PBS) e subito incubati con l'anticorpo primario. La serie di anticorpi primari, diluiti in 10mM di PBS, utilizzati sono elencati in tabella 4.1.

Il legame specifico dell'anticorpo primario è stato rilevato grazie all'uso di anticorpi secondari coniugati con specifici fluorocromi della famiglia delle cianine. Gli anticorpi secondari sono elencati in tabella 4.2.

I nuclei dei FBs in cui è stato valutato un solo marker sono stati marcati di rosso con propidio iodato (ICN Biomedicals Inc.). In parallelo sono stati eseguite immunofluorescenze come controlli negativi, dove gli anticorpi specifici primari sono stati sostituiti con gli isotipi di controllo IgG (Vector Laboratories).

Le immagini fluorescenti sono state osservate con un microscopio confocale E2000U (Nikon, Tochi, Giappone). L'acquisizione delle stesse immagini sono state catturate grazie all'uso del software C1 (Nikon) e il programma Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA).

Tabella 4.2: Elenco degli anticorpi II, coniugati con il sistema di rilevazione

Anticorpo	Specificità	Diluizione	Casa di Produzione
Cy2	anti-Mouse	600	Jackson Research, USA
Cy3	anti-Mouse	600	Jackson Research, USA
Cy2	anti-Goat	600	Jackson Research, USA
Cy3	anti-Goat	600	Jackson Research, USA
Cy2	anti-Rabbit	600	Jackson Research, USA

4.2.3 Analisi Proteiche: ELISA, cs-ELISA, Western Blotting e SDS-PAGE.

Per valutare i livelli di NGF rilasciati nel mezzo di coltura abbiamo eseguito un test ELISA chiamato "sandwich ELISA", in cui si utilizzano anticorpi specifici monoclonali fissati ad un supporto e anticorpi policlonali specifici che legano in due siti l'NGF. Il test ha una sensibilità pari a 0.5pg/mL, ed è stato eseguito seguendo il protocollo messo a punto da Weskamp e Otten [226]. Le piastre ELISA (Maxisorp NUNC 96 well plates) sono state come prima cosa incubate per una notte con l'anticorpo mouse anti-NGF (0.4μg/mL; MAB5260, R&D), affinche questo potesse fissarsi al supporto. Dopo tale trattamento, sono state preparati degli standard di NGF a concentrazione nota (da 0,15pg/mL to 1ng/mL). Gli standard insieme ai campioni opportunamente diluiti sono stati incubati a 4°C overnight. Di seguito per rilevare l'NGF legatosi in maniera specifica sono stati utilizzati rispettivamente l'anticorpo policlonale biotinilato anti-NGF (0,15μg/mL, R&D) e la soluzione di streptavidina (1/10000; Zymed, San Francisco, CA). Dopo l'aggiunta del substrato 3,3',5,5-TetraMethylBenzidine (TMB, Pierce), è stata valutata la densità ottica OD (a 450 meno 550) in ogni pozzetto, grazie all'uso del sistema "ELISA reader" (Sunrise, Tecan Systems, Inc., San Jose, CA, USA), quindi calcolati i livelli di NGF, di ogni campione, potendo costruire la curva standard di concentrazioni note di NGF in relazione agli OD relativi.

Sotto queste normali condizioni standard, non abbiamo osservato cross-reazione con Brain Derived Neurotrophic Factor (BDNF) o con NT 3/4/5. Inoltre, l'attività biologica dell'NGF

rilasciato nel medium dei FBs di OCP è stata testata separatamente usando un test biologico con cellule PC12 [227], la quale specificità è stata confermata aggiungendo l'anticorpo monoclonale neutralizzante anti-NGF (1μg/mL, R&D) ai campioni.

I livelli di citochine come l'IL-2, 5, 10 e 12, rilasciate nel mezzo di coltura dai FBs di OCP e di cataratta, sono state misurati mediante test commerciali ELISA (Biosource International, Camarillo, CA, USA) seguendo i protocolli della casa.

Per valutare gli effetti dell'NGF sull'espressione di αSMA su FBs di VKC abbiamo eseguito un particolare test ELISA, *cells surface* (cs-ELISA), che permette l'esecuzione direttamente su un monolayer di cellule, fatte crescere su piastre da 96 wells (furono piastrate circa 5x10^5/0,20mL cellule). Arrivate a confluenza, le cellule sono state trattate con diverse concentrazioni di NGF. Dopo 24h le cellule sono state lavate con HBSS contenente 0,1% di CaCL2, fissate con PFA e trattate per eliminare il segnale delle perossidasi endogene con H2O2 al 3%. Di seguito i FBs sono stati permeabilizzati con TX-PBS e poi disdratati con etanolo al 95%. Le piastre sono state quindi incubate con l'anticorpo mouse αSMA diluito 1/500 in PBS con 0.05% Tween 20 (TW). Per rilevare il legame specifico dell'alticorpo primario abbiamo utilizzato un anti-mouse coniugato con biotina (diluito 1/10000; Vector) e HRP-streptavidina (1/7000; Zymed). Abbiamo quindi sviluppato la reazione con TMB e quantificato con un lettore ELISA (Sunrise, TECAN).

Il western blot o immunofissazione è una tecnica biochimica che permette di identificare una determinata proteina in una miscela di proteine, mediante il riconoscimento da parte di anticorpi specifici. Per facilitare il riconoscimento, le miscele di proteine (30μg in Laemmli buffer), estratte da FBs di VKC, OCP e dei relativi controlli, sono state prima separate in base alle loro dimensioni (o peso molecolare) utilizzando un gel di poliacrilammide, poi soggette a elettroforesi SDS-PAGE a 160V per 3 ore (Miniprotean3, Bio-Rad); successivamente sono state trasferite su membrane di nitrocellulosa Hy-bond (apparato semi-dry blotting, Bio-Rad) a 15V per 60 min.

Ultimato il trasferimento su nitrocellulosa le membrane sono state quindi lavate con 0.05%

Tw in PBS e pretrattate con PBS contenente il 5% di latte in polvere e lasciata per 1 ora a RT. Al termine, si procede per il riconoscimento vero e proprio delle proteine, incubando le membrane con l'anticorpo specifico: MMP9 (1/700; Santa Cruz), trkA^{NGFR} (1/700), p75^{NTR} e αSMA (1/500), diluiti in PBS-TW, per 12 ore a 4°C. Il legame dell'anticorpo primario è stato localizzato mediante anticorpi coniugati HRP secondari diluiti 1 a 10000 e sviluppati mediante tecnica ECL (SuperSignal West Pico Trial, Pierce) in una Kodak imager station, visualizzata come una banda che corrisponde all'antigene di interesse. Le immagini sono state acquisite utilizzando il software 1D Kodak Image Analysis, soggetto ad analisi densitometriche, ed elaborate con Adobe Photoshop 7.0.

La normalizzazione delle proteine è stata ottenuta mediante spettrofotometro NanoDrop Tecnology ND-1000.

4.2.4 Zymografia SDS-PAGE

La zimografia è una tecnica che permette di valutare l'attività di alcune MMPs, rilasciate nel mezo di coltura delle cellule in studio. Il protocollo descritto da Berton consiste nel far separare elettroforeticamente le proteine presenti nel "medium", collettato al momento del processamento delle cellule. Ciò che differenzia questa elettroforesi da un normale western blot è il gel utilizzato per la corsa, in cui è presente un substrato delle MMPs 2 e 9 [224]. In breve, 10µL di campione, diluito con un buffer SDS, privo di β-mercaptoetanolo, (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glicerolo e 0,01% rosso fenolo), è stato prima incubato per 12 ore a 4°C e poi scaldato per 30 min. a 37°C. I campioni sono stati quindi sottoposti ad elettroforesi secondo le condizioni standard, in 10% SDS-PAGE contenente 0.1% di gelatina (Bio-Rad). Insieme ai campioni sono stai caricati il marker di pesi molecolari (6-210kDa; SERVA), il ricombinante umano latente MMP9 (92 kDa) e quello attivo (83 kDa) (Calbiochem, La Jolla, CA) come controllo positivo [224]. Lavato con una soluzione salina, 2.5% TX-PBS, per rimuovere l'SDS, il gel è stato quindi incubato a 37°C per 48 ore in una

soluzione contenente: 200 mM NaCl, 40 mM Tris-HCl e 10 mM CaCl2, pH 7.5. Di seguito, il gel è stato incubato per 3 ore con Coomassie Blue (ICN). La presenza di attività gelatinasica è stata identificata come bande trasparenti su un background blu a seguito del "destaining" con acido acetico al 7%. Le immaggini sono state acquisite mediante software1D Kodak Image Analysis su cui sono stati fatti calcoli densiometrici.

4.2.5 Analisi al Citofluorimetro: FACS

Le singole cellule, estratte e lavate con HBSS contenente Ca^{++}e Mg^{++}, sono state fissate con formaldeide al 3,7% (pH 7.4) per 1 ora. Così trattate sono state quindi nuovamente lavate e quindi direttamente processate per l'analisi al FACS o incubate in MeOH e PBS (6:1 v:v) a -20°C per 24 ore. Prima di essere analizzate, le cellule sono state lavate due volte con FACS buffer al 0,1% di saponina (0,1% NaN3 in PBS) e poi incubate con uno o due anticorpi specifici, diluiti 1 a 100 in 0,1% BSA FACS buffer. Gli anticorpi primari utilizzati sono: mouse anti-human αSMA (Novocastra, Italy); rabbit anti-human $trkA^{NGFR}$ (S. Cruz Biotech, CA, USA), mouse anti-human $trkA^{NGFR}$ (R&D, USA) e goat anti-human $p75^{NTR}$ (S. Cruz Biotech, CA, USA). Al termine dell'incubazione e dopo aver lavato le cellule per almeno due volte con FACS buffer privo di saponina, il legame specifico è stato rilevato incubando i campioni con anticorpi secondari coniugati: anti-mouse/goat FITC o Cy2, anti-mouse/rabbit PE (1:600; Jackson Research, USA). Le cellule sono state analizzate con citofluorimetro FACSCalibur usando il software Cell Quest (Becton Dickinson, Mountain View, CA).

4.3 Analisi Molecolare

4.3.1 Estrazione dell'RNA totale e conversione in cDNA

L'RNA totale è stato estratto dai monolayers confluenti (1×10^6 cells/6 well-plates or $3x10^6$ cells/T21cm^2 flasks) usando Trizol, OMNIZOL, Puregene o MirvanaParis kit. L'RNA totale è stato poi risospeso in 27μL di acqua trattata con dietil pirocarbonato (DEPC, Sigma Chemicals) e con RNase-Free DNase I (2 U/µL Turbo DNA free kit AM-1907; Ambion), in accordo con i protocolli della casa produttrice, per eliminare eventuali contaminazioni genomiche. Di seguito i campioni sono stati valutati allo spettrofotometro ($\lambda260/\lambda280>1.8$), e piccole frazioni sono state quindi fatte correre su gel di agarosio all'1% contenente bromuro di etidio (0,5 μg/mL) per controllare l'integrità del materiale nucleico estratto. Uguali quantita di RNA nrmalizzato per ogni campione sono stati usati per sintetizzare DNA a doppio filamento sulla base dei templati di RNA.

3µg total RNA sono stati trascritti in un volume finale di 20 μL, contenente 50 pM di oligo dT21-primer (Promega, Milano, Italia), 1 mM dNTP mix (Promega, Milano, Italia) e 200 U di reverse transcriptase (M-MLV, F-605L; Finnzymer, Celbio). Per favorire tale reazione i campioni sono stati sottoposti a diverse variazioni cicliche di temperature in un termociclatore programmabile: PTC100 thermocycler (MJ Research, Watertown, MA, USA).

4.3.2 Real-Time PCR

Per la reazione di amplificazione dei cDNAs è stato usato l'apparato Opticon2 MJ Research system (MJ Research). L'amplificazione è avvenuta in un volume di 20μL finali contenenti 10 μL 10xSYBR Green PCR Mix (Applied Biosystems, Foster City, CA), 3 μL di cDNA del gene target o 1μL del gene di riferimento (GAPDH o l'istone H3), e 0,5μL dei rispettivi primer (10pM, prepared by MWG, Biotech, Ebersberg, Germania). I primer specifici e di riferimento amplificati con le relative condizioni di reazione sono descritte in tabella 4.3.

Tabella 4.3: In tabella sono riassunti il nome del primer, la sequenza, la taglia, le condizioni di anniling e il numero di acceso della Genebank (G.B. Acc.no) di ogni gene valutato.

Primer	**Sequenza**	**bp**	**T°C anniling**	**G.B. Acc.no.**
NGF	f 5'-CTG GCC ACA CTG AGG TGC AT-3' r 5'-TCC TGC AGG GAC ATT GCT CTC-3'	120	55°C, 30''	BC011123
$trkA^{NGFR}$	f 5'-CAT CGT GAA GAG TGG TCT CCG-3' r 5'-GAG AGA GAC TCC AGA GCG TTG AA-3'	102	57°C, 25''	M23102
$p75^{NTR}$	f 5'-CCT ACG GCT ACT ACC AGG ATG AG-3' r 5'-TGG CCT CGT CGG AAT ACG-3'	147	57°C, 25''	AF187064
αSMA	f 5'-GAA GGA GAT CAC GGC CCT A-3' f 5'-ACA TCT GCT GGA AGG TGG AC-3'	125	55°C, 30''	BC017554
TGF-β1	f 5'-TCC TGG CGA TAC CTC AGC AA-3' r 5'-GCC CTC AAT TTC CCC TCC AC-3'	110	57°C, 25''	BC017288
TLR4	f 5'-AAT CCC CTG AGG CAT TTA GG-3' r 5'-CAG GGC TAA ACT CTG GAT GG-3'	100	60°C, 25''	HSU88880
TLR9	f 5'-TTC ATG GAC GGC AAC TGT TA-3' f 5'-GAG TGA CAG GTG GGT GAG GT-3'	102	65°C, 25''	AB045180
MMP9	5'-CCA GGA GTT CGA GGC TGT AG-3' 5'-TCC CAA ACC ACA GGA CTT TC-3'	113	60°C, 25''	BC006093
GAPDH	f 5'-CCT GAC CTG CCG TCT AGA AA-3' r 5'-ACC TGG TGC TCA GTG TAG CC-3'	111	55°C, 30''	BC013310
H3	f 5'-GCT TCG AGA GAT TCG TCG TT-3' r 5'-GAA ACC TCA GGT CGG TTT TG-3'	113	59°C, 30''	NM005324

Gli esperimenti sono stati effettuati in duplicato per ogni campione. I valori quantitativi sono stati estrapolati dai valori (Ct) ottenuti. Il valore (Ct) rappresenta il ciclo in cui l'Opticon registra il primo incremento significativo di fluorescenza. Il numero di trascritti del gene di riferimento è quantificato come controllo interno, quindi ogni campione viene normalizzato a seconda della quantità di GAPDH o H3 espresso.

Cosiderando che ogni campione contiene una uguale quantità di RNA, durante le varie fasi della reazione di PCR, le piccole variazioni di intensità di fluorescenza, dipendenti dal segnale del dye SYBR green I legato al (ds)DNA amplificato, possono essere rilevati. Al termine di ogni fase di elongazione, l'ammontare dei prodotti di PCR sono monitorati per ogni ciclo. La specificità dei prodotti viene valutata mediante l'analisi delle curve di melting di ogni campione (in duplice copia), monitorate a 60°C, 90°C.

I valori ottenuti da ogni specifico prodotto (valutato in Ct) sono espressioni relative rispetto ad un controllo interno, "housekeeping gene", confrontati con l'espressione dei campioni di controllo valutati con il software REST© [228]. I risultati sono espressi come numero di volte che il gene target incrementa o viceversa decrementa la sua espressione nei campioni in studio rispetto ai campioni di controllo. Come controllo negativo (-RT), l'amplificazione è stata fatta su un campione in cui durante la reazione di revers non è stato aggiunto l'enzima

trascriptase o in assenza di RNA totale.

4.4 Analisi Statistiche

I dati molecolari e biochimici sono stati analizzati per le loro differenze significative ($p<0.05$), utilizzando un softwere statistico StatView II (Abacus Concepts. Inc., Barkley, CA, USA). Le analisi ANOVA [229] sono state estrapolate usando il test appropriato, tukey-kramer post-hoc test.

Capitolo 5

Risultati

Il delicato processo di healing che segue un danno tissutale, indipendentemente dalle cause che lo hanno determinato, è un processo molto complesso che necessita dell'interazione e cooperazione di diversi tipi cellulari quali cellule epiteliali, stromali ed infiammatorie. La capacità di un tessuto di risolvere in un fisiologico healing è influenzato da diversi fattori come la localizzazione della lesione, il tipo di tessuto, predisposizione genetica e l'eventuale tipo di infiammazione che si instaura localmente. L'NGF, insieme ai suoi recettori, partecipa in modo attivo nel riparo fisiologico [230]. Il primo studio sull'uomo, tuttora aperto, è stato condotto su pazienti con ulcere neurotrofiche corneali a cui è stato somministrato l'NGF sotto forma di collirio [135]. I dati ottimistici prodotti da questa sperimentazione sono tuttora un esempio della capacità terapeutica, non convenzionale, dell'NGF.

Studi sperimentali su modelli animali [200] insieme a diversi dati clinici, hanno contribuito nell'ipotizzare che l'NGF possa agire direttamente sia sulle cellule strutturali che immunitarie oppure indirettamente stimolando la produzione di altri fattori pro-fibrogenici [173]. Oggi sappiamo che l'NGF oltre ad agire sulla sopravvivenza e differenziazione è anche un potente regolatore della migrazione/proliferazione delle cellule epiteliali umane, favorisce ed accelera i processi di chiusura del danno agendo sui FBs/myoFBs ed è stato inoltre dimostrato che stimoli il rilascio di TGF-β1 da FBs congiuntivali e *vice-versa* [142].

Nelle diverse fasi di questo complesso processo l'NGF e i suoi recettori sono diversamente espressi dalle cellule strutturali che partecipano al riparo del danno. Probabilmente, l'NGF modulando l'espressione dei suoi recettori potrebbe regolare la sopravvivenza/apoptosi dei myoFBs cambiando il rapporto trkA^{NGFR}/p75^{NTR} nelle varie fasi del processo riparativo, in parallelo al suo effetto sul TGF-β1 rilasciato dai myoFBs [142]. In figura 5.1 mostriamo un modello schematico che riassume il complesso "cross-talk" tra l'NGF e FBs/myoFBs durante il meccanismo riparativo di healing [229].

In questi anni, ci siamo interessati a diverse patologie oculari con un importante decorso fibrotico. In particolare abbiamo iniziato con lo studiare il rapporto tra i myoFBs presenti nelle membrane epiretiniche idiopatiche (MEI) ed NGF. La MEI, conosciuta come puker maculare, è una patologia caratterizzata dalla crescita di una membrana sulla macula e si pensa che questa condizione sia caratterizzata dalla crescita di tessuto fibrotico che contrae il tessuto retinico con conseguente distorsione della vista. Da questo studio è emerso: che le MEI sono caratterizzate dalla presenza di myoFBs che esprimono sia NGF che i suoi recettori, che i livelli di NGF sono bassi in quelle membrane in cui il livello di TGF-β1 è alto e che le cellule del vitreo mostrano un elevata espressione dei trascritti trkA^{NGFR} e p75^{NTR}, associata ad un decremento del trascritto del recettore TGF-βRII. Tutto ciò ci ha suggerito che l'NGF e i suoi recettori, espressi dai myoFBs locali, siano associati con l'evoluzione e l'attività contrattile mediata dai myoFBs delle MEIs [231].

Questo studio insieme ad altri dati sui FBs di VKC ci hanno indirizzati verso lo studio dell'NGF nel processo di remodelling, prendendo in considerazione due patologie con un diverso quadro infiammatori cronico (th1, th2), entrambe caratterizzate da un decorso evolutivo verso la fibrosi: VKC e OCP.

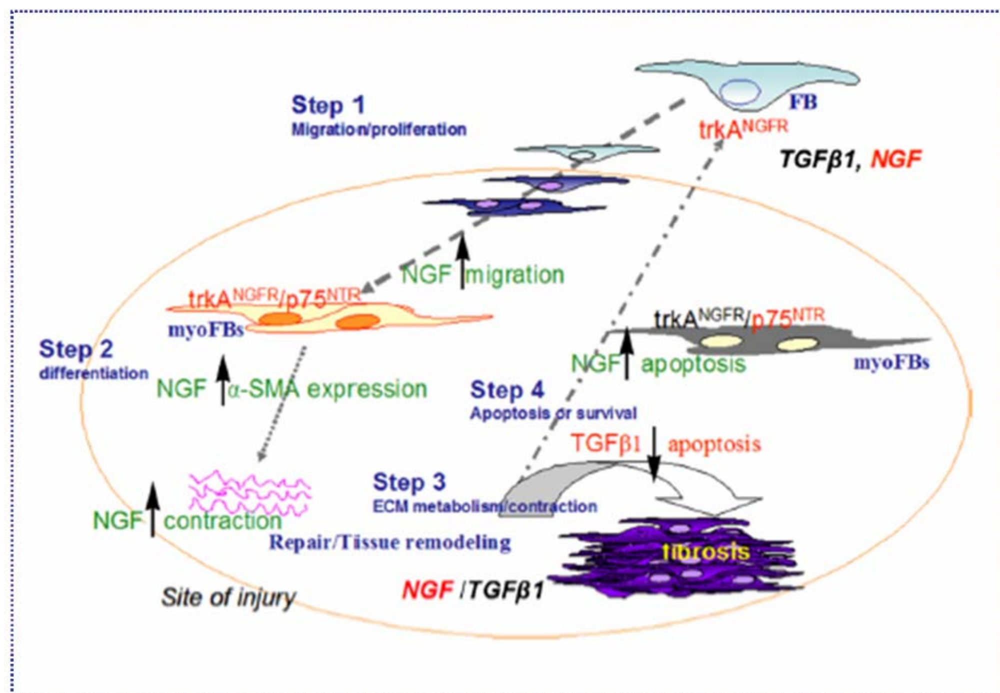

Figura 5.1: Rappresentazione schematica del complesso cross-talk tra NGF e FBs/myoFBs. In verde sono rappresentati gli effetti dell'NGF osservati in studi eseguiti in vitro. Step-1: FBs quiescenti esprimono solo trkA^{NGFR}, stimolati con NGF migrano nel sito danneggiato; step-2: differenziano in myoFBs, esprimono sia il marker αSMA che i recettori trkA^{NGFR} e p75^{NTR}; step-3: cambiamenti nel metabolismo dell'ECM, contrazione/chiusura del danno; i livelli e quindi il rapporto tra NGF e TGF-β1 determineranno poi o la morte o la sopravvivenza dei myoFBs. Sia FBs che myoFBs rilasciano NGF.

5.1 Ruolo modulatorio dell'NGF su colture primarie di FBs di VKC: Th2

I dati ottenuti in vitro mostrano che una buona percentuale di FBs congiuntivali di VKC sono differenziati in myoFBs, che rilasciano alte quantità di NGF ed esprimono entrambi i suoi recettori, associati all'espressione di αSMA [142].

5.1.1 Selezione e caratterizzazione delle biopsie di VKC utilizzate nello studio.

Abbiamo isolato FBs congiuntivali da bambini e adolescenti che presentavano sia i segni clinici che istologici di VKC. La diagnosi di VKC attiva si basa sulla storia e gli esami clinici, confermati dalla presenza di EOs nelle biopsie. La VKC viene classificata secondo uno "score" clinico che va da 0 a 3 (0= assente; 1= leggera; 2= media; 3= severa) assegnato secondo la presenza di ciascuno dei seguenti sintomi oculari: prurito, iper-lacrimazione, fotofobia e sensazione di corpo estraneo; e ciascun segno clinico: iperemia congiuntivale, iper-secrezione di muco, erosione dell'epitelio corneale e delle papille.

5.1.2 FBs di pazienti VKC esprimono NGF, trkA^{NGFR}, p75^{NTR} e αSMA.

I livelli di NGF rilasciati nel mezzo di coltura dai FBs di VKC sono significativamente superiori rispetto ai FBs di controllo (563,33±35,12 pg/mL vs. 445,67 ±10,59 pg/mL; *p<0,05). Analisi molecolari, sulle stesse cellule, hanno confermato un aumento anche del messaggero dell'NGF in FBs di VKC (Figura 5.2A).

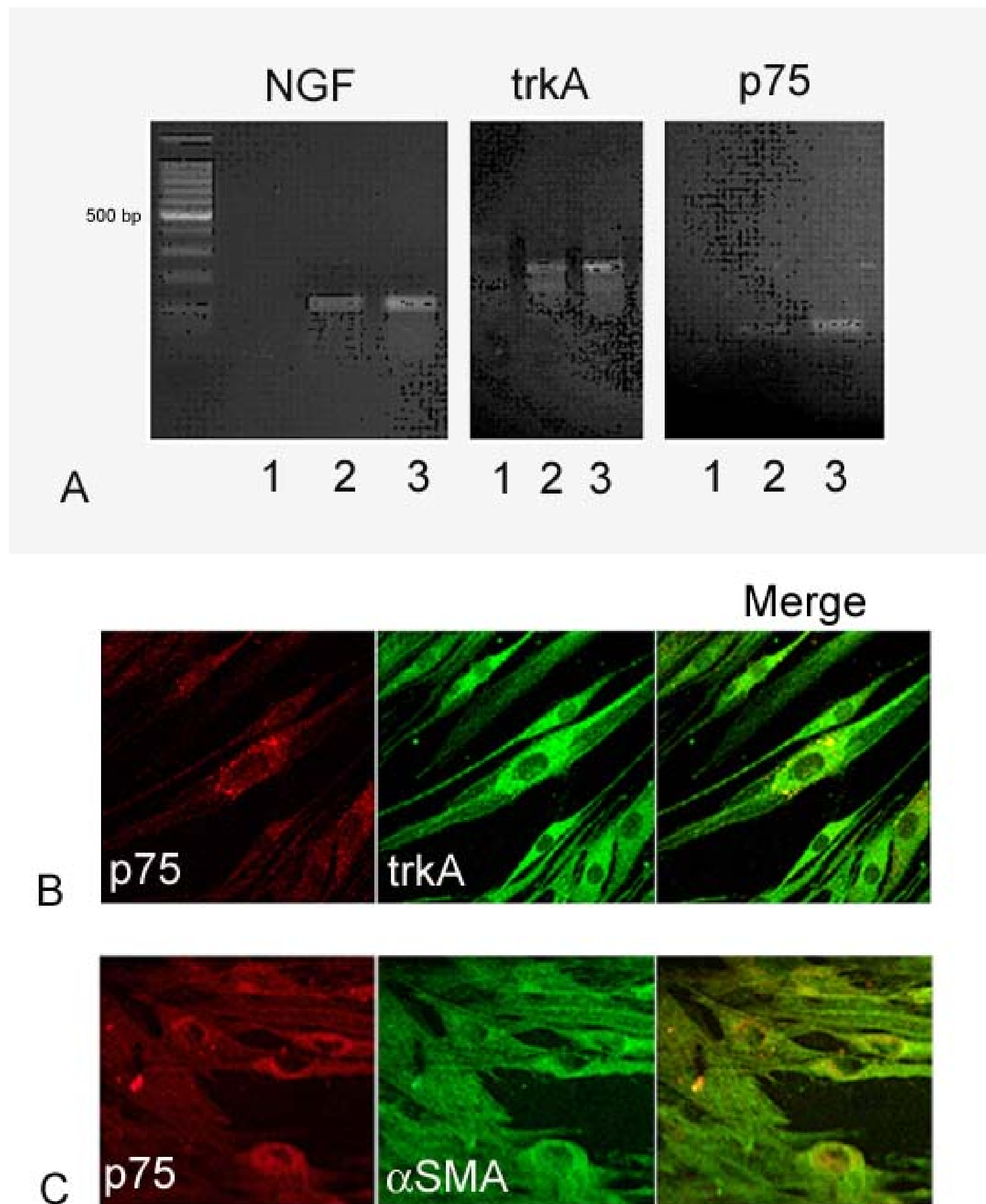

Figura 5.2: Espressine di NGF, trkA^{NGFR}, p75^{NTR} ed αSMA in FBs di VKC. A: Real-Time PCR; gel di agarosio dove sono stati corsi i prodotti di PCR, da sinistra verso destra sono mostrati gli amplificati NGF (120bp), trkA^{NGFR} (103bp) e p75^{NTR} (100bp); i numeri 1, 2 e 3 corrispondono rispettivamente ai campioni RT, FBs di controllo e FBs di VKC. In figura sono mostrati gel rappresentativi di tre esperimenti separati dove sono stati amplificati ugual quantità di cDNA. B: Microscopia al confocale, FBs di VKC esprimono sia p75^{NTR} che trkA^{NGFR} (merge, pannello di destra). C: microscopia al confocale, mostra la co-localizzazione di αSMA e p75^{NTR} in alcune cellule FBs di VKC (merge, pannello di destra).

Per quanto riguarda i recettori dell'NGF, i nostri dati mostrano che diversamente dai FBs di controllo che esprimono solo il recettore trkA^{NGFR}, i FBs di VKC li esprimono entrambi. Tuttavia, in figura 5.2AB, mostriamo anche che l'espressione di trkA^{NGFR} è "down" regolata nei FBs di VKC rispetto ai FBs di controllo (*p<0,05). Inoltre, attraverso cs-ELISA, abbiamo valutato un significativo aumento nei livelli di αSMA (0,38±0,027 OD, FBs di VKC vs. 0,232±0,046 OD, FBs ctr; *p<0,01), la cui espressione sembra co-localizzare con p75^{NTR}(Figura 5.2C) nei FBs di VKC.

5.1.3 L'NGF non modula l'espressione di αSMA in VKC-FBs

Per valutare la capacità modulatoria dell'NGF sull'espressone di αSMA, abbiamo esposto FBs di VKC a diverse concentrazioni di NGF (da 0 a 500ng/mL). Gli esami biochimici eseguiti su queste cellule, cs-ELISA e WB, dimostrano che NGF non ha effetto significativo su questo marker, p>0,05 (Figura 5.3). Contemporaneamente, abbiamo neutralizzato le stesse cellule con anticorpi verso trkA^{NGFR}o p75^{NTR}, 30 minuti prima di stimolarle alle stesse concentrazioni con NGF. Da questo secondo esperimento, abbiamo potuto verificare che l'NGF produce un significativo aumento dell'espressione di αSMA solo quando queste cellule sono neutralizzate del recettore p75^{NTR} (Figura 5.3; *p<0,05).

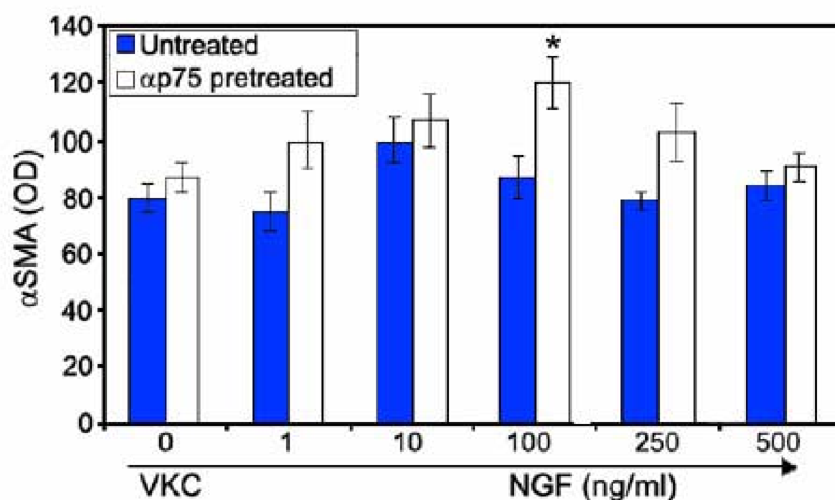

Figura 5.3: Espressione di αSMA in FBs di VKC. Trattamenti con aumentate concentrazioni di NGF non mostrava nessun effetto sui livelli di espressione di αSMA (barra blu, p>0,05). Diversamente, solo il pretrattamento di neutralizzazione con anticorpi anti-p75^{NTR} , e non quello con anti-trkA^{NGFR}, produceva cambiamenti significativi sull'espressione di αSMA (100ng/mL NGF; *p<0,05).

5.1.4 L'NGF modula l'espressione e la funzione della MMP9 in VKC-FBs

Nel processo di riparo l'ECM subisce dei cambiamenti nella sua composizione, in modo particolare ci siamo interessati alla possibilità che l'NGF potesse modulare la MMP9, la quale svolge un ruolo pilota nell'alterazione dell'ECM, guidando specificatamente il clivaggio del collagene di tipo IV, altamente espresso dai FBs di VKC [232].

Dai nostri studi risultò che l'espressione della MMP9 è modulata dall'NGF in modo dose-dipendente, ovvero solo alcune concentrazioni di NGF riuscivano ad avere un effetto significativo sulla sua espressione. Abbiamo determinato sia i livelli del trascritto che delle proteine totali ed attive di MMP9. Attraverso Real Time PCR, mostriamo che FBs di VKC trattate con 10ng/mL di NGF, sono significativamente stimolate ad aumentare di 3,92 volte la sintesi del trascritto per la MMP9 rispetto ai campioni di VKC non trattati (Figura 5.4A; *p<0,05). In figura 5.4B mostriamo un istogramma che rappresenta l'espressione della

MMP9, valutata mediante WB, dove è evidente l'aumento significativo della produzione di MMP9 dopo stimolazione con NGF, con un picco massimo alla concentrazione di 10ng/mL, associato ad un aumento dell'attività della MMP9 investigata tramite zimografia (Figura 5.4B,C).

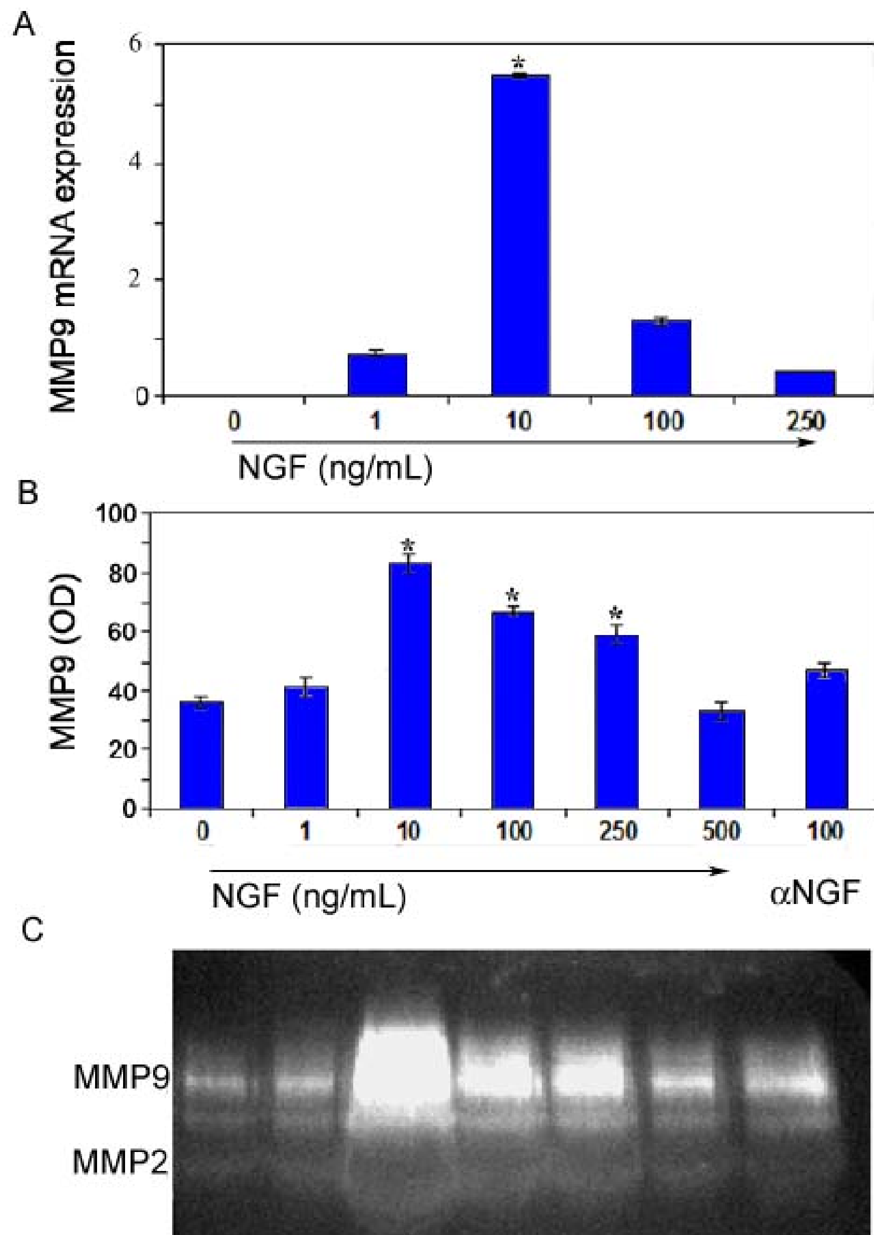

Figura 5.4: Effetto dell'NGF sull'espressione di MMP9 in FBs di VKC. A: Real-Time PCR; in figura è mostrato un aumento significativo del trascritto MMP9 da parte dei FBs di VKC trattati con 10ng/mL di NGF (*$p<0{,}05$). B, C: rispettivamente WB e Zimografia; l'aumento del trascritto era accompagnato anche da un aumento nei livelli di sintesi della proteina e del suo rilascio nella forma attiva nel mezzo di coltura (* $p<0{,}05$).

5.2 Ruolo modulatorio dell'NGF su una patologia di tipo Th1: OCP

I dati mostrano che nella congiuntiva di pazienti affetti da OCP sono presenti FBs differenziati in myoFBs che rilasciano e utilizzano NGF. Inoltre è emerso che l'espressione di αSMA e p75^{NTR} sia strettamente legata allo stadio patologico e/o all'evoluzione della malattia.

5.2.1 Selezione e caratterizzazione delle biopsie di OCP utilizzate nello studio.

La formazione e la localizzazione di autoanticorpi a livello della membrana basale dell'epitelio congiuntivele innesca il fenomeno infiammatorio che è alla base della malattia. I tessuti dei pazienti selezionati, che mostravano un sospetto diagnostico clinico di OCP, confermato dall'indagine microscopica, sono quelli che noi abbiamo utilizzato per la messa in coltura dei FBs; in Figura 5.5 è mostrata una immunofluorescenza positiva alle IgGAM a livello della membrana basale, confrontata con un controllo di cataratta.

Nel pemfigoide vi è un rimodellamento della matrice extracellulare della sostanza propria del tessuto congiuntivale. Sono state proposte diverse classificazioni della patologia, ma nessuna è molto soddisfacente a causa dell'estrema variabilità clinica. Tuttavia abbiamo cercato di suddividere i diversi pazienti in due gruppi principali, uno stadio iniziale e uno avanzato di patologia, sulla base dello stato della fibrosi tissutale, dell'epitelio congiuntivale, dalla presenza o meno di cellule mucipare, edema, flogosi mostrata dall'analisi istochimica degli stessi pazienti selezionati (Figura 5.6).

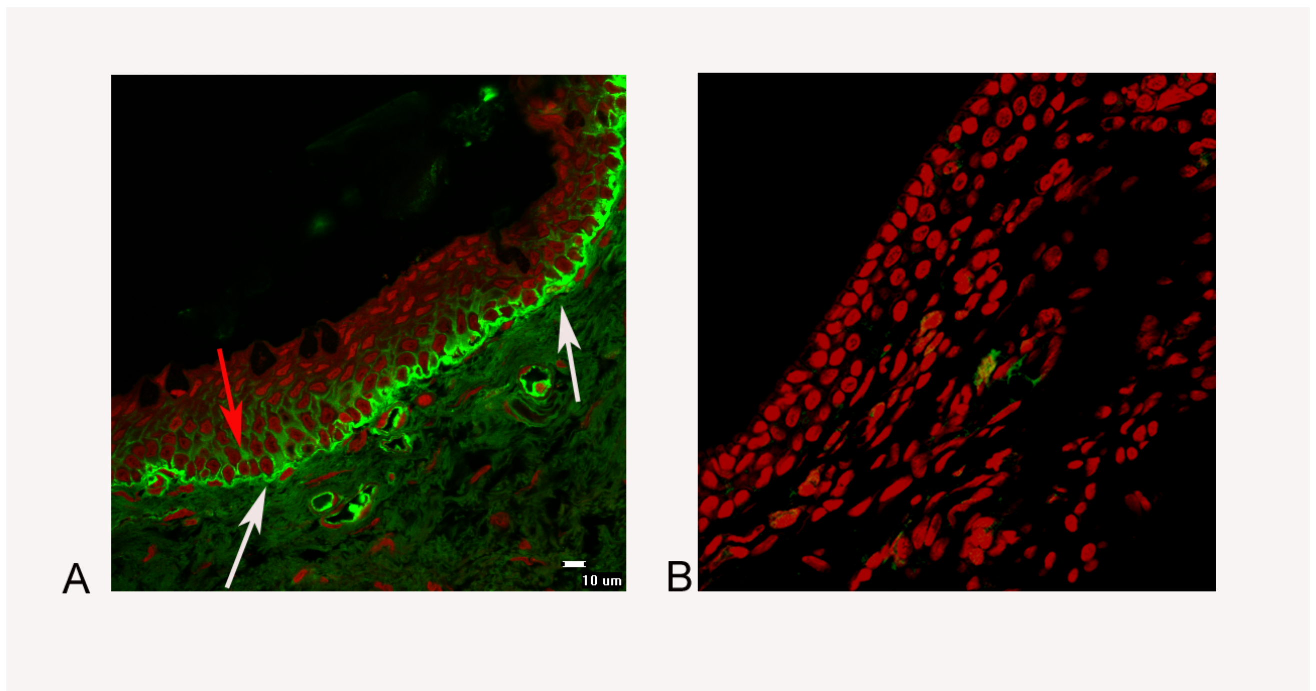

Figura 5.5: Diagnostica per OCP. A: immunofluorescenza positiva per IgGAM (Cy2/verde) a livello della membrana basale congiuntivale (freccia bianca) e in alcuni punti della sostanza intercementante dell'epitelio stesso (freccia rossa), ottenuto da un paziente ad uno stadio avanzato di OCP (x60). B: immunofluorescenza negativa per IgGAM (Cy2/verde) effettuata su un tessuto congiuntivale, ottenuto da un paziente di cataratta, con la medesima procedura del tessuto patologico (x60). In rosso sono evidenziati i nuclei delle cellule, marcati con propidio.

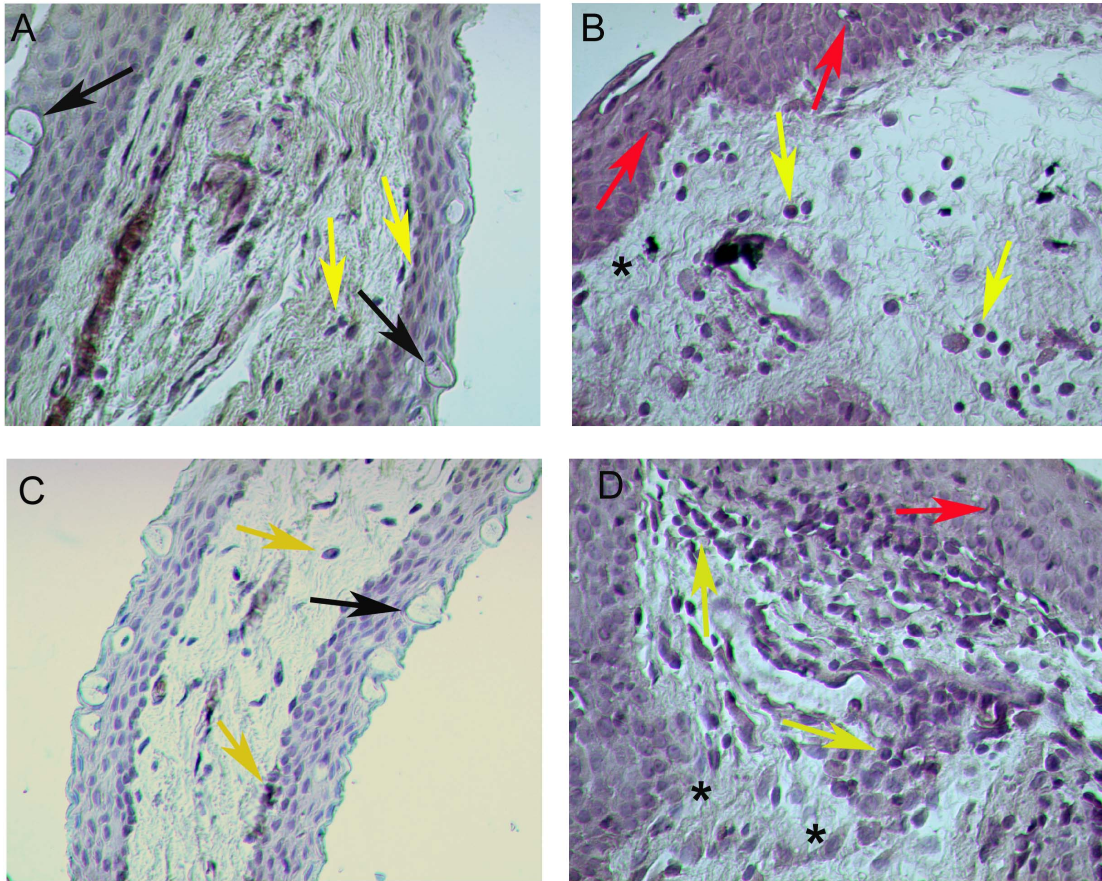

Figura 5.6: E&E. A,C: due sezioni congiuntivali, rappresentative una fase infiammatoria cronica iniziale di OCP, in cui è evidente un epitelio apparentemente normale, ricco di cellule mucipare (frecce nere), scarsa flogosi di tipo linfocitario (freccie gialle), lieve edema e fibrosi (x40). B,D: due sezione congiuntivali di due pazienti in avanzato stadio di OCP, dove è evidente una maggior flogosi di tipo linfocitario e granulocitario (freccia gialla) con evidente esocitosi (freccia rossa), epitelio privo di cellule mucipare, presenta strati pavimentosi. Evidente edema, soprattutto a livello dell MB, ed attiva fibrosi (evidenziata con asterischi) (x40).

5.2.2 Espressione di αSMA e NGF nei tessuti di OCP

E' noto che il cronico insulto infiammatorio, che si stabilisce a livello della membrana basale congiuntivale, porta nel tempo ad un danno tissutale con conseguente fallimento del tentativo riparativo che evolve in fibrosi. Ci siamo appunto chiesti se i FBs, che normalmente sono le cellule responsabili degli eventi di healling tissutale, migrino a livello del danno e si trasformano in myoFBs anche nel loci danneggiati dei tessuti congiuntivali di OCP. In figura 5.7 mostriamo la presenza di αSMA nello stroma dei tessuti congiuntivali, a conferma della presenza di myoFBs a livello della zona lesa.

La dimostrazione della presenza dei recettori trkA^{NGFR} e p75^{NTR}, a livello dello sroma congiuntivale di OCP [134], ci ha portato a riflettere sulla possibilità che questo tessuto potesse essere recettivo all'NGF, sia endogeno che esogeno. Considerando inoltre, che nelle patologie infiammatorie allergiche l'NGF è stato intensivamente studiato per il suo ruolo immunomodulatorio e valutato molti dati di letteratura che suggerivano un'influenza dell'NGF sui FBs/myoFBs, abbiamo cercato di capire se e quali cellule della congiuntiva di OCP esprimessero NGF, e se ci fossero delle differenze con i normali controlli di cataratta che potessero essere correlati con la patologia o con il fenotipo αSMA. Mostriamo in Figura 5.8, la diversa espressione dell'NGF, che in una normale congiuntiva è largamente espresso dalla maggior parte delle cellule epiteliali mentre è praticamente assente nello stroma. Diversamente nel tessuto patologico l'NGF è pressoché scarso nell'epitelio e più espresso nello stroma (Figura 5.8).

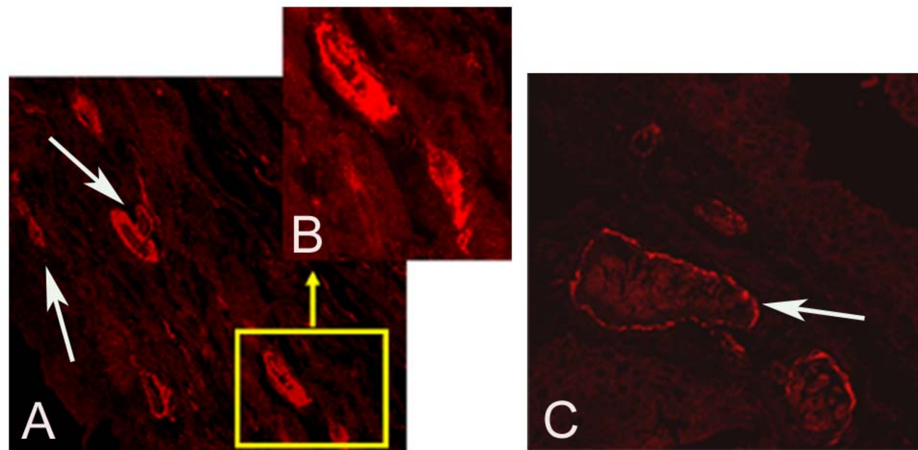

Figura 5.7: Immunofluorescenza per αSMA (Cy3/rosso), eseguito su tessuti congiuntivali, ottenuti da pazienti affetti da OCP e cataratta. A: la marcatura positiva per αSMA su biopsia di OCP, indicata con frecce bianche, si accumula in zone cellulari che stanno ad indicare la presenza di cellule come i myoFBs o miociti (x60). B: ingrandimento di cellule simili ai myoFBs, presenti nella foto A. C: marcatura di αSMA su biopsia di cataratta, positiva a livello dei vasi, per la presenza dei miociti (freccia bianca; x40)

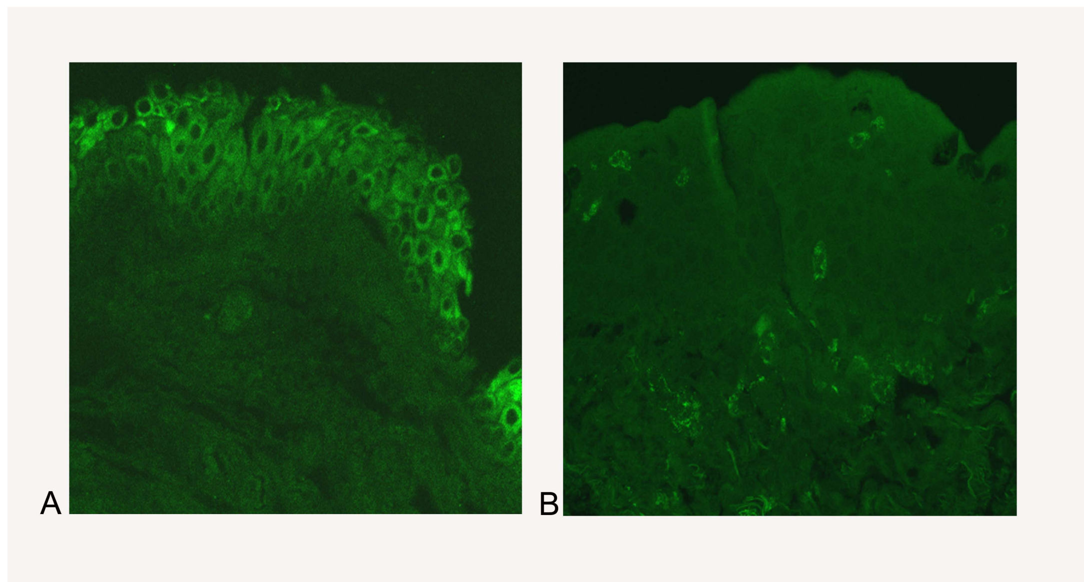

Figura 5.8: Espressione dell'NGF a livello congiuntivale. A: immunofluorescenza su tessuto congiuntivale ottenuto da biopsie di cataratta, l'NGF (Cy2/verde) è distribuito su tutta la superficie epiteliale e assente nello stroma. B: tessuto patologico di OCP, presenta una scarsa espressione di NGF a livello epiteliale mentre aumenta nello stroma (x40).

5.2.3 FBs di OCP esprimono il marker per i myoFBs: αSMA

La presenza del marker fenotipico αSMA nei tessuti congiuntivali di pazienti affetti da OCP, ci ha fatto ipotizzare che i myoFBs, osservati nel tessuto, avessero origine dai FBs stromali, stimolati a differenziare a causa del danno alla MB e il particolare quadro infiammatorio del paziente. Tutte le nostre attenzioni sono state quindi indirizzate nello studio in vitro dei FBs congiuntivali di OCP.

In figura 5.9 mostriamo sia con immagini al confocale che con istogrammi ottenuti da analisi al citofluorimetro, l'aumento di espressione di αSMA in FBs di OCP rispetto ai FBs di cataratta, a conferma della nostra ipotesi iniziale. Gli esami biochimici sono stati eseguiti parallelamente all'analisi molecolare Real-Time PCR, da cui è emerso che in FBs di OCP, il numero di copie del trascritto per questo gene aumentava di 14 volte rispetto al numero normalmente prodotto dai FBs di cataratta.

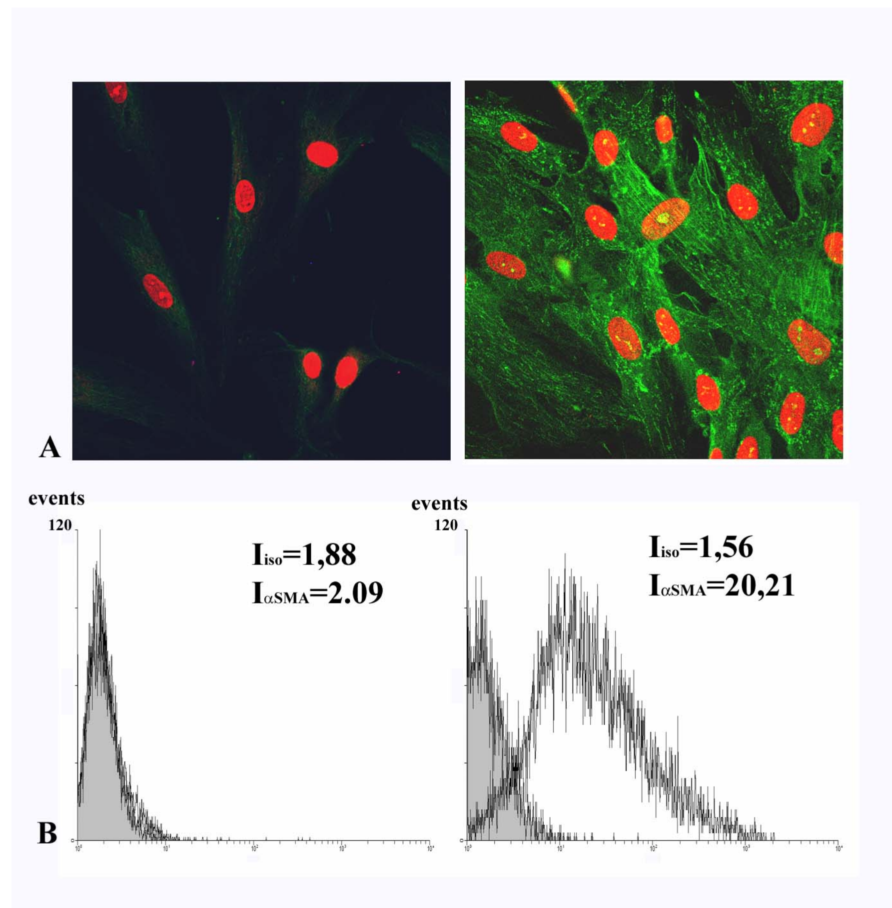

Figura 5.9: Espressione di αSMA in FBs di OCP. A: un immagin, rappresentativa per ogni gruppo, di immunofluorescenza per αSMA (Cy2/verde) su FBs congiuntivali di cataratta (sinistra) e OCP (destra); i nuclei sono stati evidenziati in rosso, marcati con propidio, (x60). B: i due grafici mostrano l'intensità di fluorescenza I (Cy2) per la proteina αSMA in FBs di cataratta (sinistra) e in FBs di OCP (destra); tali dati sono stati ottenuti su 5000 eventi contati.

5.2.4 FBs di OCP sintetizzano/rilasciano NGF ed esprimono i recettori trkA^{NGFR}/p75^{NTR}

Le immagini al confocale mostrato una maggior espressione dell'NGF a livello dello stroma congiuntivale di OCP confrontato con il tessuto di cataratta. L'aumento di NGF nello stroma potrebbe esser dovuto a più fattori concomitanti come la presenza di molte cellule infiammatorie e/o di FBs/myoFBs stimolati nella sintesi di NGF. Abbiamo quindi misurato in vitro, i livelli del trascritto e della proteina rilasciata nel mezzo di coltura dai FBs congiuntivali di OCP e cataratta. I FBs di OCP producevano un numero di copie del trascritto NGF 5 volte superiore a quello dei FBs di cataratta e rilasciavano anche una maggiore quantità di proteina nel mezzo di coltura (4200±900pg/mL vs. 2200±640pg/mL; $p>0{,}05$).

La seconda domanda che ci siamo posti era se queste cellule fosse in grado di utilizzare l'NGF rilasciato e quindi rispondere localmente attraverso i suoi recettori. Mediante analisi al confocale ed al citofluorimetro, abbiamo quindi valutato l'espressione di trkA^{NGFR} e p75^{NTR} sia in FBs di OCP che di cataratta. In cellule di OCP, p75^{NTR} appariva fortemente espresso rispetto a trkA, il cui segnale appariva solo in alcune cellule, debolmente co-espresso con p75^{NTR}. Al contrario, in FBs di cataratta trkA^{NGFR} era maggiormente espresso rispetto al debole segnale di p75^{NTR} (Figura 5.10AB).

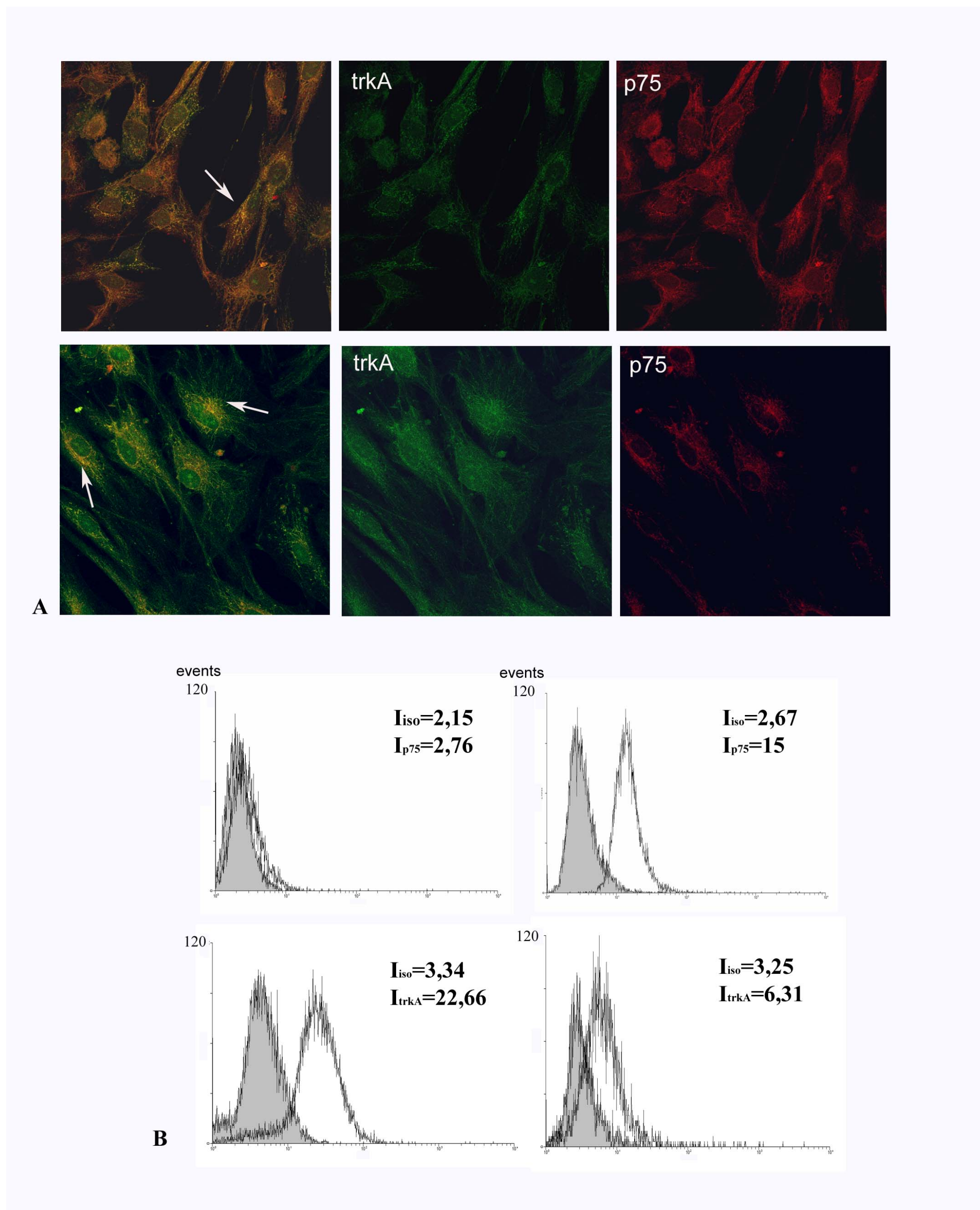

Figura 5.10: Espressione dei recettori trkA^{NGFR} e p75^{NTR}, in FBs di OCP e cataratta. A: un immagine rappresentativa di ciascun gruppo, di doppia e singola immunofluorescenza per trkA^{NGFR} (Cy2/verde) e p75^{NTR}(Cy3/rosso) su FBs di OCP (in alto) e di cataratta (in basso); le frecce bianche indicano la co-localizzazione di espressione dei due recettori. B: i grafici, ottenuti dalle analisi al citofluorimetro, mostrano l'intensità di fluorescenza I per la proteina p75^{NTR} (in alto: FITC) e trkA^{NGFR} (in basso: PE) in FBs di cataratta (sinistra) e di OCP (destra); tali dati sono stati ottenuti su 5000 eventi contati.

5.2.5 L'espressione di αSMA co-localizza con il recettore p75^{NTR}, in alcuni FBs di OCP

Per verificare l'ipotesi che l'NGF potesse influenzare la differenziazione dei FBs in myoFBs abbiamo studiato la distribuzione dei recettori trkA^{NGFR}, p75^{NTR} ed il marker αSMA in FBs di OCP. Attraverso wester blot abbiamo potuto osservare che l'aumento dei livelli proteici di αSMA in OCP era accompagnato da un'alta espressione del recettore non specifico p75^{NTR}, mentre trkA^{NGFR} era presente a livelli inferiori rispetto i controlli (Figura 5.11A). L'analisi al confocale ci ha permesso di osservare che mentre il recettore trkA^{NGFR} non era espresso dalle stesse cellule che esprimevano αSMA, il recettore p75^{NTR} co-localizzava con il marker dei myoFBs (Figura 5.11B,C), suggerendo una sua implicazione con tale fenotipo.

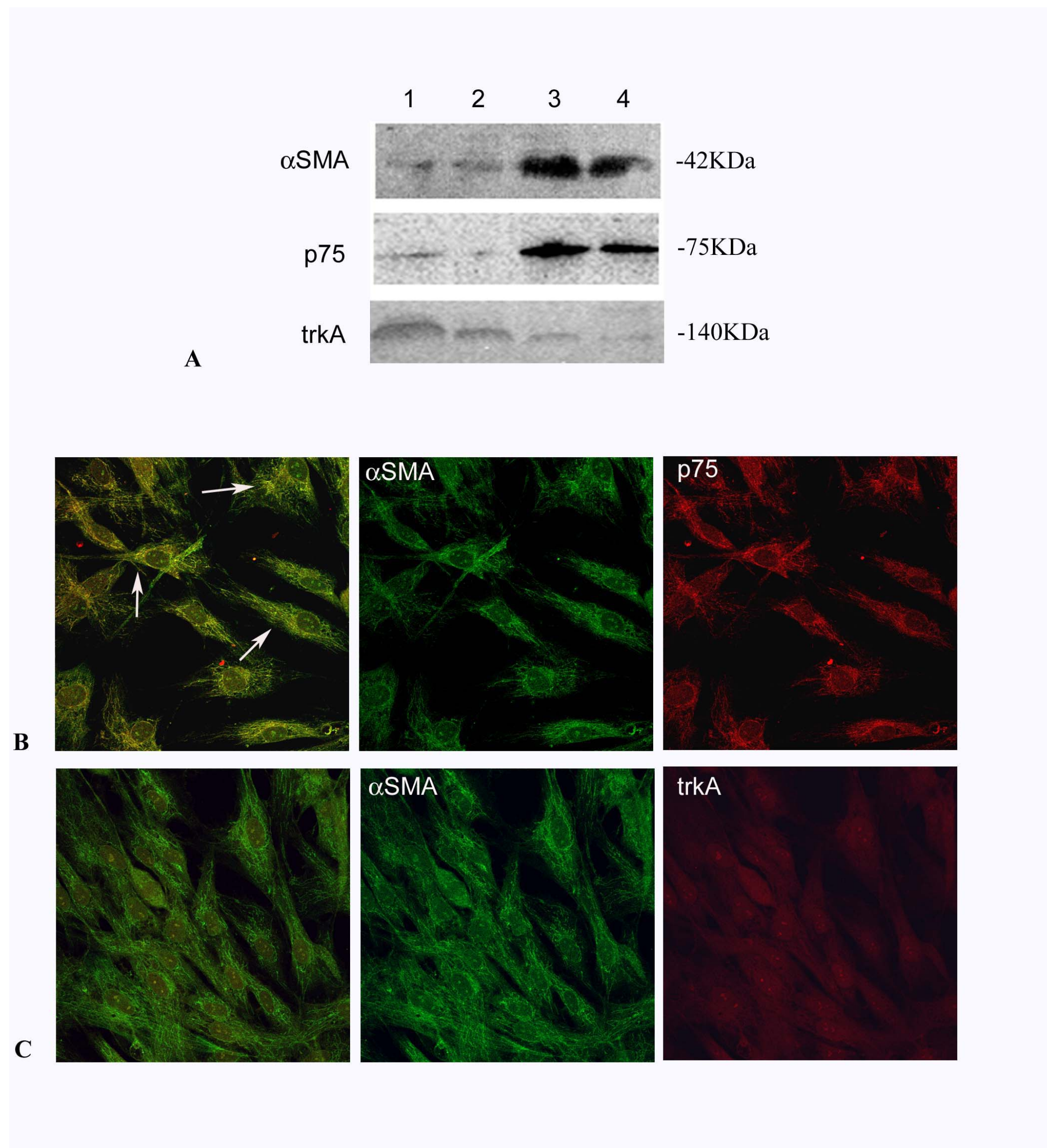

Figura 5.11: Co-localizzazione tra αSMA ed i recettori dell'NGF, in FBs di OCP. A: Western blot per αSMA (42 KDa), $p75^{NTR}$ (75 KDa) e $trkA^{NGFR}$ (140 KDa); con i numeri 1,2 sono indicati due campioni rappresentativi di controllo, con 3 e 4 due campioni rappresentativi di OCP. B: un immagine rappresentativa, di doppia e singola immunofluorescenza per αSMA (Cy2/verde) e $p75^{NTR}$(Cy3/rosso) su FBs di OCP, (x60); le frecce bianche indicano la co-localizzazione dei due marker. . C: un immagine rappresentativa, di doppia e singola immunofluorescenza per αSMA (Cy2/verde) e $trkA^{NGFR}$ (Cy3/rossa) su FBs di OCP.

Tuttavia, abbiamo notato che l'espressione dei due recettori era strettamente legato allo stato patologico della malattia. Per questo motivo abbiamo deciso di classificare i FBs di OCP a seconda dello stato istopatologico dei tessuti, da cui FBs erano stati ottenuti, e per il fenotipo degli stessi FBs:

1. "early" fibrosi: il tessuto presentava un quadro infiammatorio attivo e una debole fibrosi; inoltre, la maggior parte delle cellule ottenute da queste biopsie presentavano una forma conforme al normale FBs;

2. "severe" fibrosi: il quadro infiammatorio non più attivo era accompagnato da un'alterazione del tessuto, le cellule ottenute da queste biopsie presentavano una forma non sempre allungata ed affusolata ma stellata. Inoltre, queste cellule crescevano più lentamente rispetto gli altri FBs di OCP e cataratta.

Nei FBs classificati come "early" i trascritti (mRNA) di trkA^{NGFR}, p75^{NTR} ed αSMA aumentavano rispetto ai normali FBs ma solo nei "severe" FBs l'aumento di αSMA, p75^{NTR} (aumentano 9 $\pm$ 0,4 e 5 volte $\pm$ 0,5 rispettivamente; **p<0,0001) ed il decremento di trkA^{NGFR} diventavano significativi (decresce 4 volte $\pm$ 1; *p<0,05) (Figura 5.12A). I dati proteici, valutati attraverso l'analisi al citofluorimetro da cui abbiamo ottenuto e calcolato la "$\triangle$Mean Fluorescence Intensity" ($\triangle$ MFI), confermavano, in linea di massima, i dati molecolari. Le differenze erano sostanzialmente due: la prima che p75^{NTR} aumentava in modo significativo già nei "early" FBs (5,5$\pm$0,35 vs. 0,4$\pm$0,07; **p<0,0001), la seconda che trkA^{NGFR} aumentava, invece di diminuire come il suo trascritto, nei "severe" FBs (0,46$\pm$0,23 vs. 3,8$\pm$1; **p<0,001). Mentre, allo stesso modo del messaggero, αSMA aumentava significativamente nei "severe" FBs (8,03$\pm$1,7 vs. 0,05$\pm$0,0035,**p<0,001; Figura 5.12B).

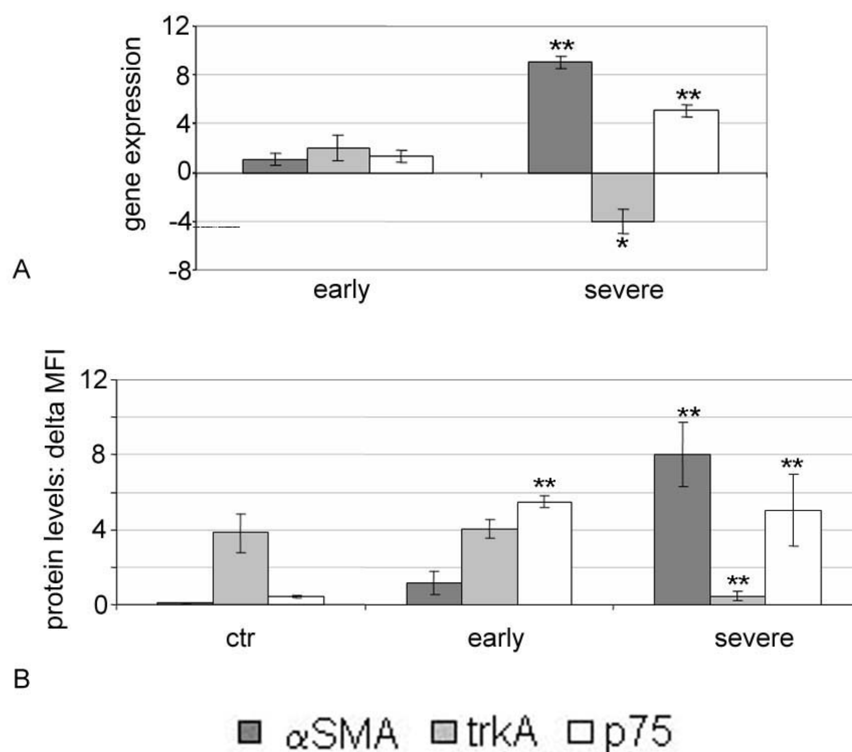

Figura 5.12: Espressione di αSMA, trkA^{NGFR} e p75^{NTR} in "early" e "severe" FBs di OCP. A: Real-Time PCR, istogramma che mostra il numero di volte che i trascritti di questi geni aumentano/diminuiscono, nei due gruppi di FBs di OCP rispetto a quelli dei FBs di cataratta; αSMA: grigio scuro, trkA^{NGFR}: grigio chiaro, p75^{NTR}: bianco. B: FACS, istogramma che mostra la $\triangle$ MFI, nei FBs di cataratta e nei due gruppi di OCP; αSMA: grigio scuro,trkA^{NGFR}: grigio chiaro, p75^{NTR}: bianco.

5.2.6 Ruolo modulatorio dell'NGF sul fenotipo di FBs/myoFBs di OCP

Caratterizzati i FBs congiuntivali di OCP, per studiare il possibile ruolo modulatorio dell'NGF sull'espressione dei due recettori trkA^{NGFR}, p75^{NTR}, il marker αSMA ed il fattore pro-fibrogenico TGF-β1, abbiamo sottoposto queste cellule a dosi crescenti di NGF (0.1, 1, 10, 50 e 100ng/mL) per 24h. L'analisi molecolare di queste cellule, ha mostrato un lieve

incremento del trascritto trkA^{NGFR} nei FBs denominati "early" quando stimolati con NGF, rispetto ai FBs non trattati (p>0,05; dati non mostrati); diversamente, è diminuito, anche se in modo non significativo il numero di copie dei trascritti p75^{NTR} αSMA e TGF-β1 nelle stesse cellule. Nei FBs in avanzato stato fibrotico "severe" si nota un'inversione di tendenza nella trascrizione dei geni per p75^{NTR}, αSMA e TGF-β1, in cui i trascritti sono aumentati (incrementano alla concentrazione 10ng/mL rispettivamente:8,3±4, 3±2,5, 1,4±0,8; p>0,05), mentre trkA^{NGFR} alla stessa concentrazione è diminuita (FBs OCP: -3±1,2 volte vs. FBs di OCP non trattati; p>0,05).

I dati molecolari sono stati parallelamente confrontati con quelli proteici tramite il calcolo delle △ MFI, estrapolate dai dati al citofluorimetro. Solo la dose di 10ng/mL di NGF, diversamente dalle altre, è riuscito a stimolare la sintesi di trkA^{NGFR} in FBs di OCP in "early" fibrosi (6,4±0,5 vs. 3,1±0,7; *p<0,05; Figura 5.13A), mentre non abbiamo osservato cambiamenti significativi in "severe" fibrosi. Diversamente, p75^{NTR} ed αSMA sono significativamente diminuiti nelle stesse cellule alla dose di 10ng/mL di NGF (0,5±0,3 vs. 5,5±1,1 *p< 0,05; 0,2±0,15 vs. 1,15±0,3 *p< 0,05, rispettivamente; Figura 5.13B,C).

Circa il 94.91% dei FBs di OCP ottenuti da pazienti che mostravano una fibrosi "early", sono risultati essere trkA^{NGFR} positivi. Il 57.24% di queste cellule in particolare co-esprime con il recettore p75^{NTR}, mentre solo lo 0,18% presenta unicamente p75^{NTR} (Figura 5.13D). Dopo stimolazione con 10ng/mL di NGF, queste stesse cellule hanno subito dei cambiamenti nell'espressione dei due recettori: in dettaglio il 72,76% dei FBs esprime solo trkA^{NGFR}, mentre il p75^{NTR} è diminuito fino ad arrivare al 24.37%, di cui il 24,12% co-espresso con trkA^{NGFR} (Figura 5.13E). In FBs di OCP, ottenuti da pazienti che mostravano una fibrosi "severa", invece era più alta la percentuale di cellule p75^{NTR} positive (∼36%) rispetto al trkA^{NGFR} (∼1,4%). Inoltre, a seguito del trattamento con NGF(10ng/mL), non sono stati osservati significativi cambiamenti nella percentuale di espressione dei due recettori.

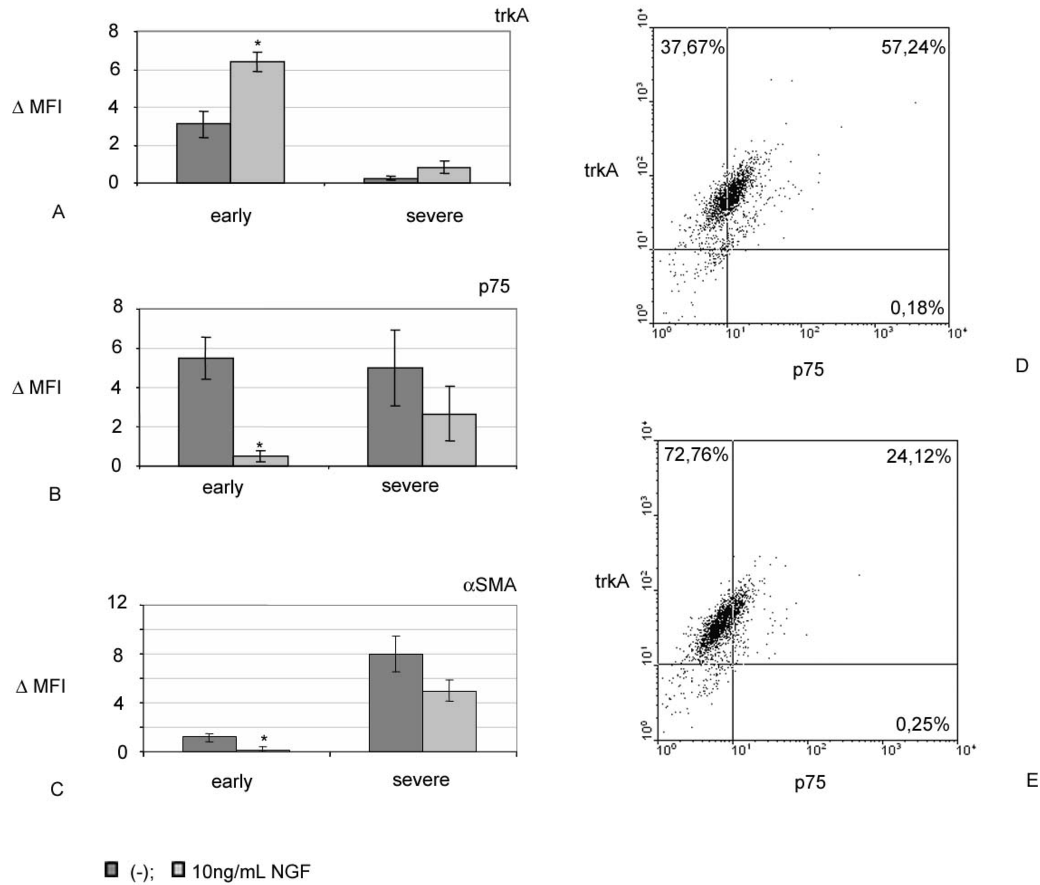

Figura 5.13: Effetti di NGF su trkA^{NGFR}, p75^{NTR} e αSMA in FBs di OCP.A,B,C: istogrammi delle $\triangle$ MFI, rispettivamente dei marker trkA^{NGFR}, p75^{NTR} e αSMA ottenute dall'analisi al citofluorimetro dei FBs di OCP ottenuti da pazienti che presentavano "early" o "severe" fibrosi. D,E: dot plot che riporta la percentuale di espressione o co-espressione dei recettori trkA^{NGFR} e p75^{NTR}, rispettivamente in FBs di OCP, ottenuti da pazienti che presentavano una "early" fibrosi, non trattati e trattati con 10ng/mL di NGF .

5.2.7 L'NGF non modula l'espressione e la funzione della MMP9 in OCP-FBs

In FBs di VKC, abbiamo precedentemente dimostrato che l'espressione della MMP9 è modulata in modo dose-dipendente dall'NGF. Considerando quindi l'importanza di questa MMP

nei processi di remodelling, abbiamo studiato gli effetti dell'NGF anche sui FBs di OCP.

I nostri dati mostrano che l'espressione della MMP9 in FBs di OCP è variabile all'interno dello stesso gruppo patologico e rispetto ai controlli di cataratta. In particolare i FBs "severe" rilasciano più MMP9 attiva rispetto gli altri FBs, sia di OCP "early" che di cataratta. Tuttavia non abbiamo osservato cambiamenti nell'attività della MMP9 quando le cellule di OCP sono state stimolate con NGF (Figura 5.14ABC).

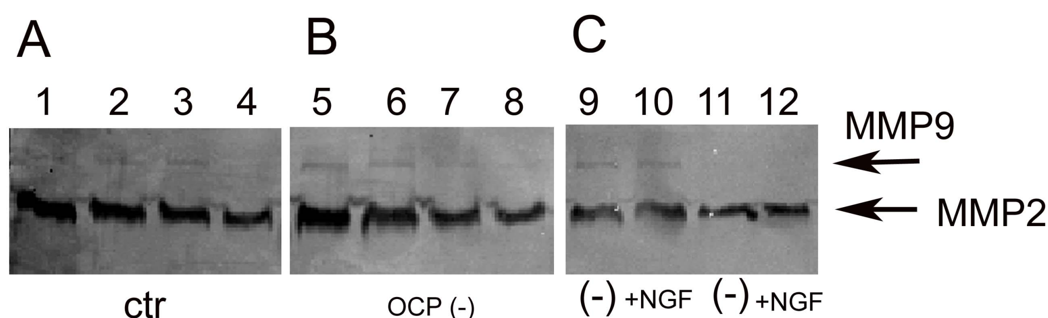

Figura 5.14: Attività delle MMPs in FBs di OCP. A,B: zimografia, di 4 campioni rappresentativi per ogni gruppo; i numeri 1,2,3 e 4 corrispondono a campioni di cataratta; 5 e 6 ai campioni di FBs di OCP "severe", mentre 7 e 8 ai FBs di OCP "early". C: un campione rappresentativo per ogni gruppo di FBs di OCP trattati e non con NGF : i numeri 9 (gruppo "severe") e 11 (gruppo "early") sono FBs non trattati, mentre i numeri 10 e 12 corrispondono rispettivamente agli stessi campioni OCP ma stimolati con 10ng/mL di NGF.

5.3 Ruolo dell'NGF nella modulazione dell'infiammazione Th1

Le popolazioni di linfociti T helper sono distinguibili sulla base del profilo citochinico, come osservato nelle diverse sotto-popolazioni linfocitarie T: Th1, Th2 e Th3. Tuttavia, nelle risposte infiammatorie croniche si osserva spesso la predominanza di uno dei sottotipi Th1 o Th2. L'equilibrio tra i due subsets nel corso di una reazione immunitaria è il principale fattore responsabile dello sviluppo di una risposta protettiva o di una sequela patologica. In seguito all'esposizione a diverse citochine, i Th0 differenziano in Th1 o Th2 a seconda

del microambiente. Ad esempio, l'IFNγ agisce in modo paracrino/autocrino nel favorire la differenziazione in Th1, inibendo al contempo la proliferazione dei Th2; similmente IL-4 ed IL10 agiscono in modo paracrino/autocrino nella differenziazione in Th2, inibendo al tempo stesso la proliferazione dei Th1. Per tale motivo, una risposta immunitaria che comincia a svilupparsi in una delle due direzioni si polarizza sempre più fino ad arrivare a una situazioni cronica. Come precedentemente descritto, le patologie croniche infiammatorie sono caratterizzate da un disequilibrio Th1/Th2 in favore di uno dei due sottotipo linfocitari. L'OCP, definita una patologia di tipo Th1, presenta alcuni caratteri Th2, come la presenza di EOs e mastociti, alti livelli di IL-4, IL-5 ed altri fattori come "Macrophage Migration Inhibitory Factor" (MIF) e TGF-β1 nella congiuntiva infiammata [233, 234]. L'esordio è prettamente Th1 ma ad un certo punto manifesta caratteristiche tipiche di una risposta Th2. Ciò dovuto al rilascio di IL-4 da parte dei Treg, che inibisce l'azione dell'IFNγ, favorendo uno *switch* Th2 [235]. I FBs che vengono attivati dall'iniziale risposta infiammatoria Th1 per ristabilire il buon healing tissutale, potrebbero in fasi tardive contribuire al processo, come dimostrato da Foster e coll. [234, 236].

Per studiare in dettaglio questi fenomeni, abbiamo deciso di valutare i livelli basali di alcune citochine, considerate pro/anti-infiammatorie della risposta Th1 (pro-: IL-2, IL-12, IFNγ; anti-: IL-10, IL-4, TGF-β), prodotte dai FBs di OCP, e la capacità dell'NGF di modularne il rilascio nel terreno di coltura.

Rispetto ai FBs di cataratta, i FBs di OCP in fase "early" rilasciano nel "medium" significative quantità di IL-2, 4 e TGF-β1 (% di rilascio vs. ctr, rispettivame: 3403%±346, **p<0,001; 609%±200, *p<0,05; 458%±13, **p<0,001), al contrario dei FBs di OCP "severe" che invece tendono a rilasciarne in quantità inferiori e variabili (Figura 5.15A,B). L'IFN-γ che è basso in OCP "early" incrementa in modo significativo nei "severe" (% di rilascio vs. ctr: 434%±11; **p<0,001). Non abbiamo osservato cambiamenti significativi nel rilascio delle citochine IL-12 e 10 da parte dei FBs di OCP rispetto i controlli di cataratta (Figura 5.15A,B).

A seguito delle stimolazione con NGF (1,10,50ng/mL), abbiamo notato una diversa risposta dei FBs di OCP sia in fase "early" che "severe" . I livelli dell'IL-2 e 4 sono diminuiti in modo significativo (% di rilascio vs. ctr. IL-2 a 1, 10 e 50ng/mL:-97%±3, **P<0,001; -74%±26, *p<0,05; -26%±10, p>0,05. IL-4 a 1, 10 e 50ng/mL:-64%±21, p>0,05; -87%±5,2, **p<0,001; -85%±7,3, **p<0,001) , mentre quelli del TGF-β1 sonorisultati essere influenzati dalla dose di NGF (% di rilascio vs. ctr a 1, 10 e 50ng/mL rispettivamente: 6%±3, p>0,05; 30%±20, p>0,05; -99%±4) in FBs di OCP "early" (Figura 5.15C,D). I livelli di IL-10 risultano aumentati negli stessi FBs con un picco significativo a 10ng/mL di NGF (% di rilascio vs. ctr: 70%±10, *p<0,05). Al contrario nei FBs "severe" l'IL-2 è aumentata con un picco significativo alla concentrazione di 10ng/mL di NGF (% di rilascio vs. ctr: 500%±64, **p<0,001), mentre i livelli rilasciati di IL-4 e TGF-β1 risultano diminuiti significativamente raggiungendo il picco minimo alla dose di 10ng/mL (% di rilascio vs. ctr: rispettivamente -88%±5, **p<0,001; -99%±10, *p<0,05; Figura 5.15C,D). L'IL-10 è invece è risultata aumentata nelle stesse cellule in modo non significativo (p>0,05; Figura 5.15C,D).

Non abbiamo osservato cambiamenti significativi nel rilascio delle citochine IL-12 e IFN-γ da parte dei FBs di OCP stimolati con NGF (Figura 5.15A,B).

5.4 Effetto dell'NGF sull'immunità innata: TLRs.

I "Toll like receptors" (TLRs) sono considerati recettori sentinella dell'immunità innata perchè per primi riconoscono proteine o DNA/RNA batterico, fungineo e virale, attivando una serie di risposte che producono mediatori e citochine pro-infiamatore nella prima fase antimicrobica [237]. L'abilità dei Tolls nel guidare il cross/talk tra le due risposte immunitarie, innata ed adattativa, sta nel fatto che cellule immuno competenti che per prime entrano in contatto con patogeni esprimono specifici TLRs. L'abilità dei Tolls nel guidare il cross/talk tra le due risposte immunitarie, innata ed adattativa, sta nel fatto che cellule immuno competenti che per prime entrano in contatto con patogeni esprimono specifici TLRs.

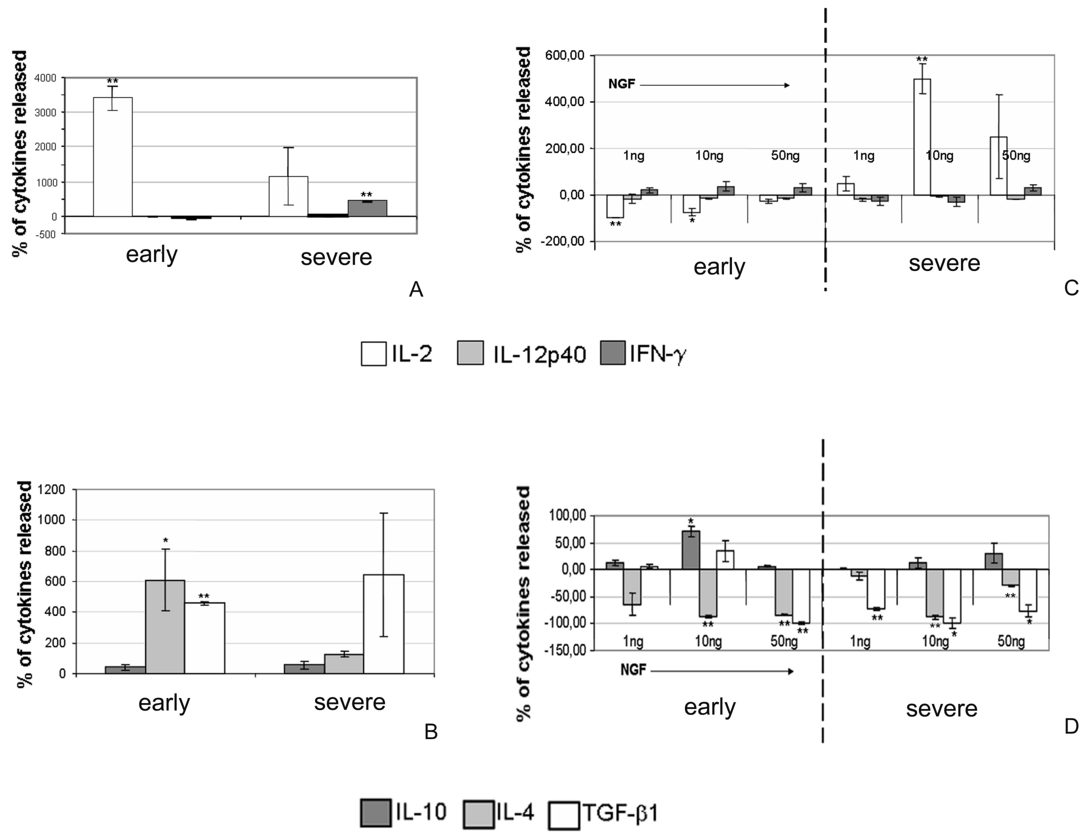

Figura 5.15: Livello di citochine, pro e anti-infiammatorie, rilasciate dai FBs di OCP trattati e non con NGF. A,B: Istogramma ELISA, rappresentativo dei livelli di citochine pro (A) o anti (B) -infiammatorie rilasciate dai due gruppi di FBs di OCP, valutati come % di espressione rispetto i livelli delle stesse citochine rilasciate dai FBs di controllo. C,D: Istogramma ELISA, rappresentativo l'effetto dell'NGF sui livelli di citochine, pro (C) o anti (D) -infiammatorie, rilasciate dai FBs dei due gruppi di OCP, trattati con diverse dosi di NGF, valutati come % di espressione rispetto ai livelli delle stesse citochine rilasciate dai FBs di OCP non trattati.

Recentemente è stata proposta l'ipotesi che l'attivazione dei TLRs possa contribuire all'istaurarsi di una bilanciata risposta Th1/2 e che il mal funzionamento di questo meccanismo possa portare ad una risposta immunitaria alterata che può evolvere fisiopatologicamente. In linea con questa idea sono state fatte numerose ricerche per indagare il ruolo della flora commensale e i TLRs nell'omeostasi delle mucose. Questa teoria esamina il ruolo della flora batterica nell'istaurarsi della fibrosi in quei tessuti dove l'equilibrio del biofil viene perturbato e cellule strutturali, come i FBs, sono attivati costitutivamente nell'overproduzione di fattori e citochine profibrogenici [238, 239, 240]. In particolare, la superficie oculare, che è costitutivamente esposta ai patogeni, esprime i TLRs sia a livello dell'epitelio congiuntivale che corneale (Figura 5.16). Tuttavia, in alcuni casi infettivi ed in particolare in casi di allergia, sembra che questa espressione sia alterata [241, 237]. Viene quindi naturale ipotizzare che controllando i TLRs e il microambiente non solo si possa guidare la risposta infiammatoria ma anche le conseguenze, a volte patologiche come il rimodellamento non fisiologico.

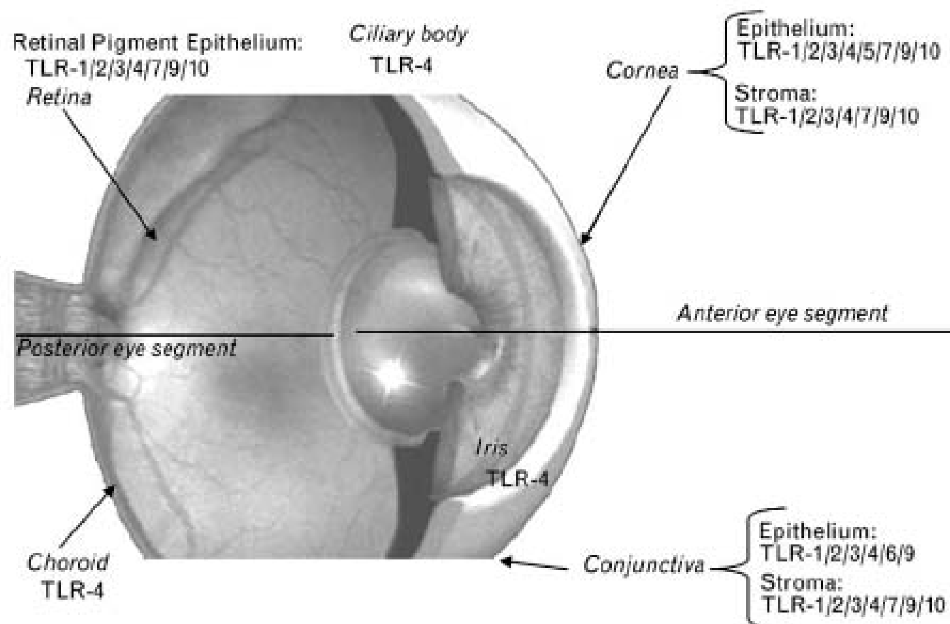

Figura 5.16: Rappresentazione grafica della distribuzione dei TLRs nell'occhio

L'origine infiammatoria dell'OCP non è conosciuta, tuttavia non si esclude un'infezione

microbica, che possa innescare, insieme ad un alterato meccanismo di difesa, la sequela di eventi che porterebbero alla fibrosi. Noi in particolare abbiamo studiato la distribuzione di espressione di due tipi di TLRs: 4[1] e 9[2], che sono quelli trovati alterati nella congiuntiva di pazienti affetti da VKC [241]. Con maggior attenzione abbiamo investigato se i FBs fossero più o meno sensibili agli stimoli diretti da parte dei microbi, responsabili dei cambiamenti del microambiete, e se l'NGF potesse intervenire sull'equilibrio immunitario della risposta innata.

Abbiamo quindi determinato in vitro l'espressione di questi due recettori nelle due patologie in studio sia per valutarne le condizioni basali che gli effetti dell'NGF. In tessuti di VKC l'espressione dei due recettori è stata già precedentemente valutata. Bonini e collaboratori hanno osservato un'espressione localizzata a livello dello stroma di entrambi i "Toll" [241]. Nella congiuntiva di pazienti di OCP i recettori TLR4 e 9 sono risultati co-localizzare sia a livello della MB che nello stroma, con evidenti accumuli cellulari comprendenti sia cellule strutturali che immunitarie (Figura 5.17A). Vista la positività stromale, abbiamo quindi marcato anche i FBs di OCP e di VKC, che sono risultati entrambi positivi per i due TLRs (Figura 5.17B,C,D). Dati molecolari, hanno confermato che i FBs di OCP tendono ad esprimere maggior mRNA dei TLR4 e 9, al contrario dei FBs di VKC che invece risultano esprimerene meno rispetto i rispettivi controlli (Figura 5.17E,F). Stimolazioni con NGF non risultavano cambiare i normali livelli di TLR4 in FBs di OCP, mentre il messaggero del TLR9 è diminuito in modo significativo alla concentrazione di 1ng/mL di NGF ($-13,8 \pm 1,3$; $^{**}p<0,001$, Figura 5.17D), [242]. Diversamente in FBs di VKC, il trattamento con NGF aumentava TLR9 mentre diminuiva in modo significativo l'espressione del TLR4 nelle stesse cellule.

[1] Toll espresso sulla superficie cellulare, riconosce costituenti lipopolisaccaridici (LPS) della parete batterica.

[2] Toll espresso sulla membrana endosomiale, riconosce motivi nucleici

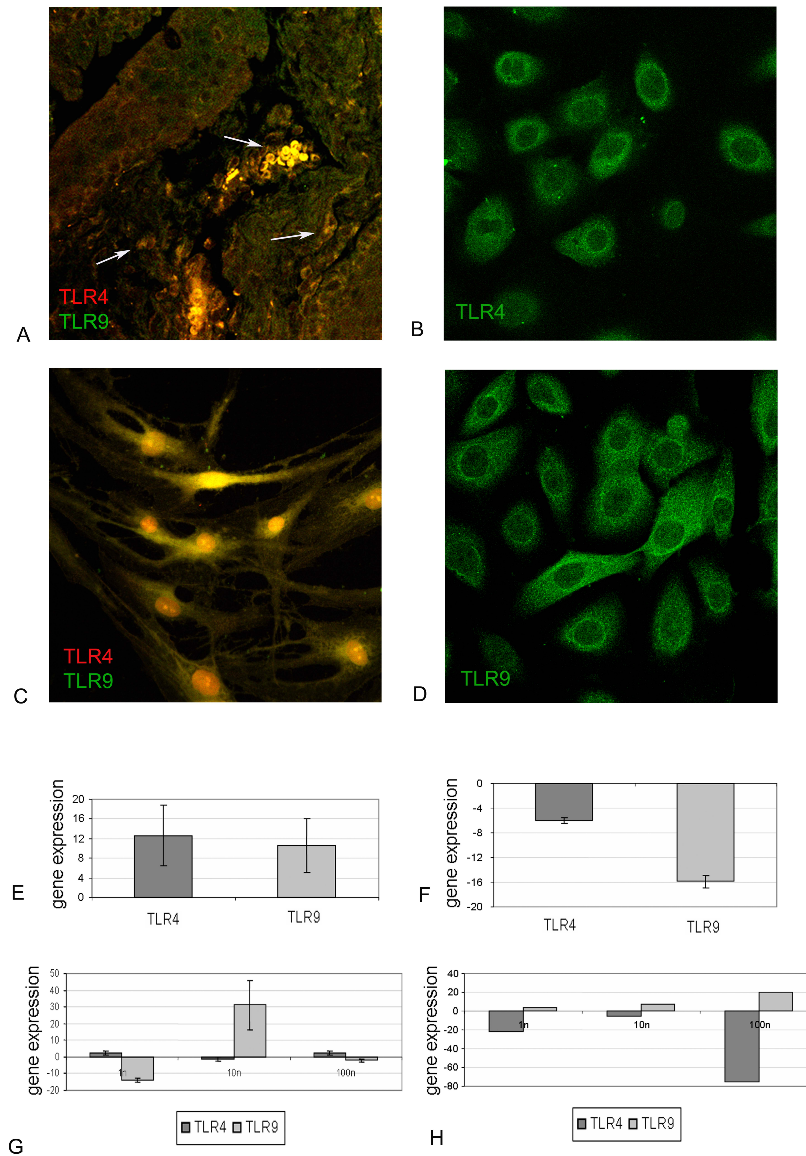

Figura 5.17: Espressione dei TLR4/9 in FBs di OCP e VKC, trattati e non con NGF. A: immunofluorescenza per TLR4 (Cy3/rosso) e TLR9 (Cy2/verde) su tessuto di congiuntiva di OCP; le frecce bianche indicano la co-localizzazione dei due recettori (x40). B:immunofluorescenza per TLR4 (Cy2/rosso) in FBs di VKC (x60). C: immunofluorescenza per TLR4 (Cy3/rosso) e TLR9 (Cy2/verde) in FBs di OCP (x60). D:immunofluorescenza per TLR9 (Cy2/verde) in FBs di VKC (x60). E-F: istogramma rappresentante il numero di volte che il trascritto TLR4 (grigio scuro) o TLR9 (grigio chiaro) aumenta/diminuisce in FBs di OCP (E) o VKC (F) rispetto al trascritto dei due recettori in FBs di controllo. G-H: istogramma rappresentante il numero di volte che il trascritto TLR4 (grigio scuro) o TLR9 (grigio chiaro) aumenta/diminuisce in FBs di OCP o VKC a seguito del trattamento con NGF (1ng/ml; 10ng/mL; 100ng/mL).

5.5 Effetto dell'NGF su cheratociti, "like" FBs, non patologici.

Caratterizzato il legame tra l'NGF e i FBs/myoFBs in termini di "Tissue Remodelling" in diverse situazioni patologiche, in cui è risultato che sia l'NGF che i suoi recettori, non solo sono alterati in tutte queste condizioni, ma influenzano anche tale processo dipendentemente dalla patologia, ci siamo chiesti come agisse l'NGF in un ambiente "standardizzato":

1. Direttamente sulla differenziazione FBs/myoFBs?
2. A livello della proliferazione?
3. A livello della migrazione?
4. A livello dell'ECM?
5. Indirettamente via TGF-β1?

Per rispondere ad ognuno di questi punti, abbiamo utilizzato FBs non patologici, altrimenti alterati, per valutare gli effetti dell'NGF in condizioni normali. Inoltre, dal momento che l'NGF contribuisce, in modo signibficativo, nel buon healing delle ulcere corneali, decidemmo di usare proprio i cheratociti corneali.

5.5.1 NGF induce la differenziazione in FBs-cheratociti in myoFBs

I cheratociti-FBs nel corso di un danno tissutale differenziano in myoFBs [243]. Queste cellule esprimono entrambi i recettori per NGF [244]. Quando abbiamo quindi stimolato con diverse dosi di NGF i cheratociti, hanno mostrato una riorganizzazione del citoscheletro, morfologicamente simile a quella dei myoFBs, e un'induzione nell'espressione di αSMA ($p<0,01$; Figura 5.18ABC). Come contro prova, a favore dell'effetto differenziativo dell'NGF, abbiamo trattato le stesse cellule con 1μg/mL di anticorpo anti-NGF, prima della stimolazione (alla

concentrazione in cui avevamo ottenuto la massima risposta), per abolirne l'effetto (Figura 5.18A).

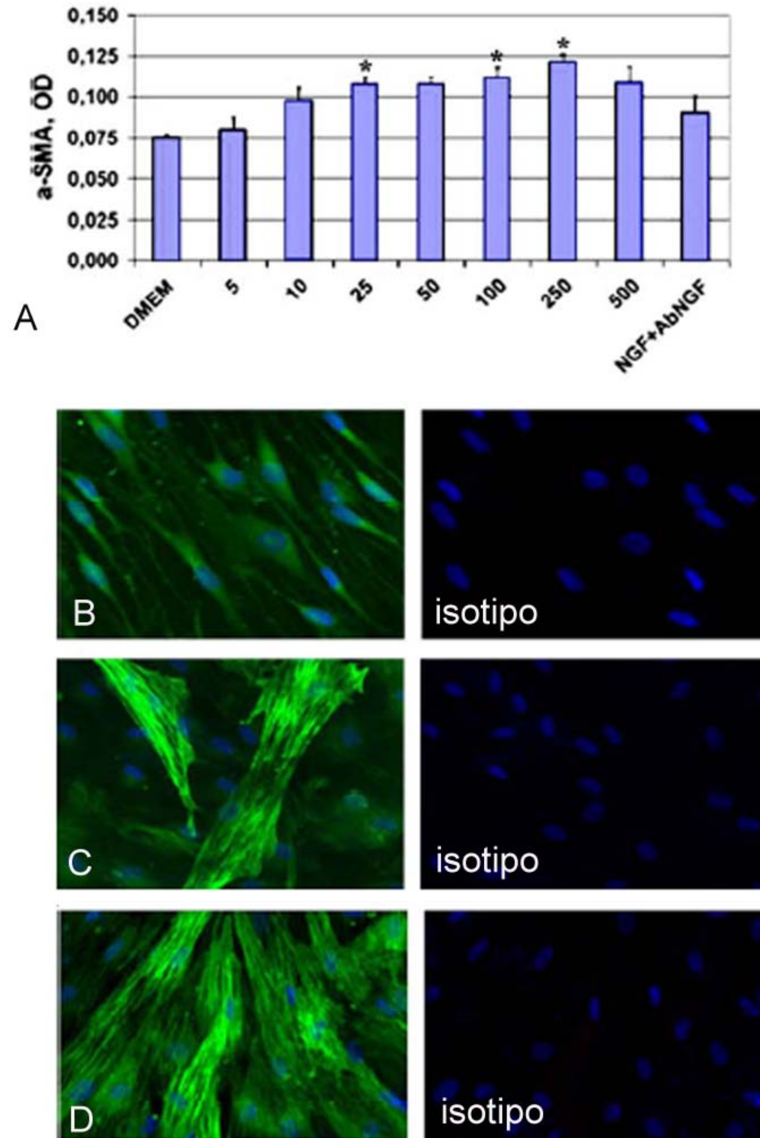

Figura 5.18: Effetto dell'NGF sull'espressione del marcher αSMA in cheratociti corneali. A: istogramma dove sono rappresentati gli OD per αSMA, ottenuti dal test cs-ELISA, dei campioni di cheratociti, a sei giorni dalla stimolazione con diverse concentrazioni di NGF (*p<0,05). B, C, E: Immunofluorescenza per αSMA (anti mouse Cy2/verde) di cheratociti trattati rispettivamente con solo medium, o con 50ng/mL di NGF o 15ng/mL di TGF-β1, i nuclei delle cellule sono stati marcati con toto3,(x40).

5.5.2 La proliferazione dei FBs-cheratociti non è influenzata da NGF

Abbiamo utilizzato due metodiche diverse per misurare la capacità dell'NGF di indurre proliferazione sui cheratociti: come prima cosa abbiamo testato la vitalità delle cellule mediante test di esclusione con Tripan blu e poi abbiamo eseguito gli esami di proliferazione sia mediante un'immuno-istochimica per il marker nucleare di replicazione Ki67 che tramite incorporazione di Timidina marcata H^3. In figura 5.19A mostriamo il mancato effetto dell'NGF sulla proliferazione dei cheratociti.

5.5.3 L'NGF aumenta la migrazione dei FBs-cheratociti verso il "taglio-danno"

Il processo di migrazione dei cheratociti verso il danno è un processo essenziale durante "l'healing". Stimoli sia a basse concentrazioni (10ng/mL) fino ad arrivare a 250ng/mL di NGF sono risultati stimolare un significativo aumento della migrazione di queste cellule verso e oltre la linea dove noi avevamo provocato un taglio 24 ore prima (*$p>0,05$). Diversamente e concorde con dati di letteratura, non ha procurato nessun effetto migratorio la stimolazione con TGF-β1 [243].

5.5.4 L'NGF stimola la contrazione del gel 3D di collagene

La contrazione del collagene, che è la proteina predominante dell'ECM, è un'altro importante evento che interviene nel processo riparativo. Le concentrazioni 50, 100 e 250ng/mL di NGF hanno indotto un significativo aumento della contrazione del gel quando abbiamo stimolato i cheratociti (circa 9×10^4 cellule) che erano stati fatti crescere su una matrice tridimensionale (3D) di collagene (*$p<0,05$; Figura 5.19C). Il trattamento a 50ng/mL di NGF ha avuto lo stesso effetto del TGF-β1, utilizzato come controllo positivo [243].

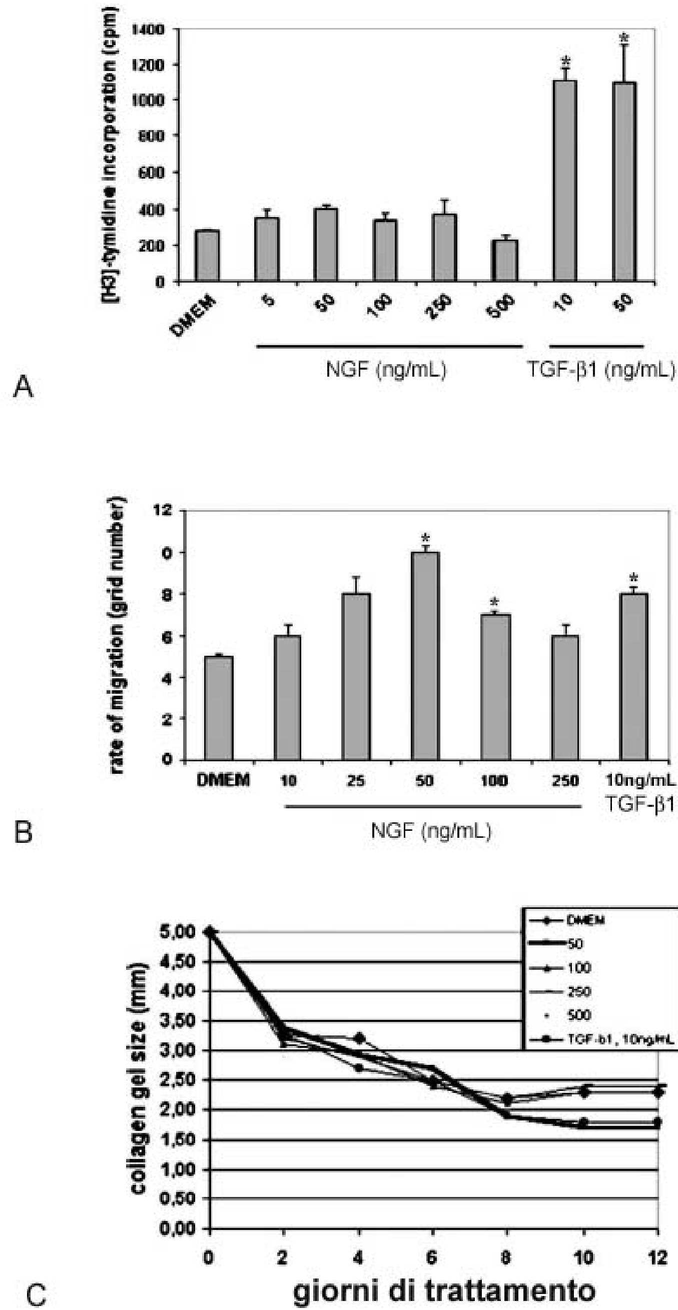

Figura 5.19: Effetto dell'NGF sulla proliferazione e migrazione dei cheratociti e sul collagene. A: istogramma ottenuto dal test di proliferazione mediante incorporazione di Timidina marcata H^3 su cheratociti trattati e non con NGF o TGF-β1. B:istogramma ottenuto dal test per la migrazione cellulare eseguito su cheratociti trattati e non con NGF o TGF-β1. C: Diagramma rappresentativo degli effetti dell'NGF o del TGF-β1 sulla contrazione del collagene da parte dei cheratociti.

5.5.5 Il TGF-β1 e l'NGF sono reciprocamente stimolati in FBs-cheratociti

Per valutare se l'effetto dell'NGF potesse esser dipendente dal TGF-β1 abbiamo sottoposto i cheratociti ad alcune prove. Come prima cosa, per capire se i due fattori stimolassero il loro rilascio reciproco, abbiamo trattato i cheratociti con diverse dosi di NGF o TGF-β1. In Figura 5.20A e B mostriamo due risultati da test ELISA che mostrano uno il significativo rilascio di TGF-β1 da cellule stimolate con NGF, confrontato con cellule non stimolate (*$p<0{,}05$ a tutte le concentrazioni di NGF con un picco massimo a 100ng/mL), e l'altro il rilascio significativo di NGF da cellule stimolate con TGF-β1 (*$p<0{,}05$, alle concentrazioni: 5 e 10ng/mL). I dati biochimici sono stati confermati anche da esami molecolari, Real-Time PCR. 250ng/mL di NGF hanno aumentano il trascritto del TGF-β1 di 1.04 volte e diminuito il prorio di 70 volt, rispetto ai non trattati. Allo stesso modo TGF-β1 ha incrementato il trascritto di NGF di 63.03 volte e di 1.61 il proprio. Per valutare la specificità di entrambi i fattori sulla differenziazione dei cheratociti in myoFBs abbiamo trattato le cellule con 250ng/mL di NGF da solo o insieme all'anticorpo anti-TGF-β1(250ng/mL), *vice versa* altre cellule sono state stimolate con 15ng/mL di TGF-β1 da solo o insieme all'anticorpo anti-NGF (500ng/mL), oppure con entrambi i fattori (250ng/mL di NGF e 15ng/mL di TGF-β1). La Figura 5.20C mostra come la neutralizzazione di NGF o del TGF-β1 non abbia abolito il loro effetto sull'espressione di αSMA. Inoltre, quando i due fattori sono stati somministrati insieme non sono stati osservati effetti aditivi o sinergici degli stessi. Diversamente dagli esami biochimici, le valutazioni molecolari in Real Time PCR di queste cellule hanno mostrato che a seguito della neutralizzazione del TGF-β1, la stimolazione con NGF diminuiva il trascritto di αSMA di 1,22 volte rispetto ai normali livelli. Al contrario, il trattamento con TGF-β1 dopo la neutralizzazione dell'NGF aumentava il trascritto di αSMA di 1,06 volte rispetto ai normali livelli (Figura 5.20D).

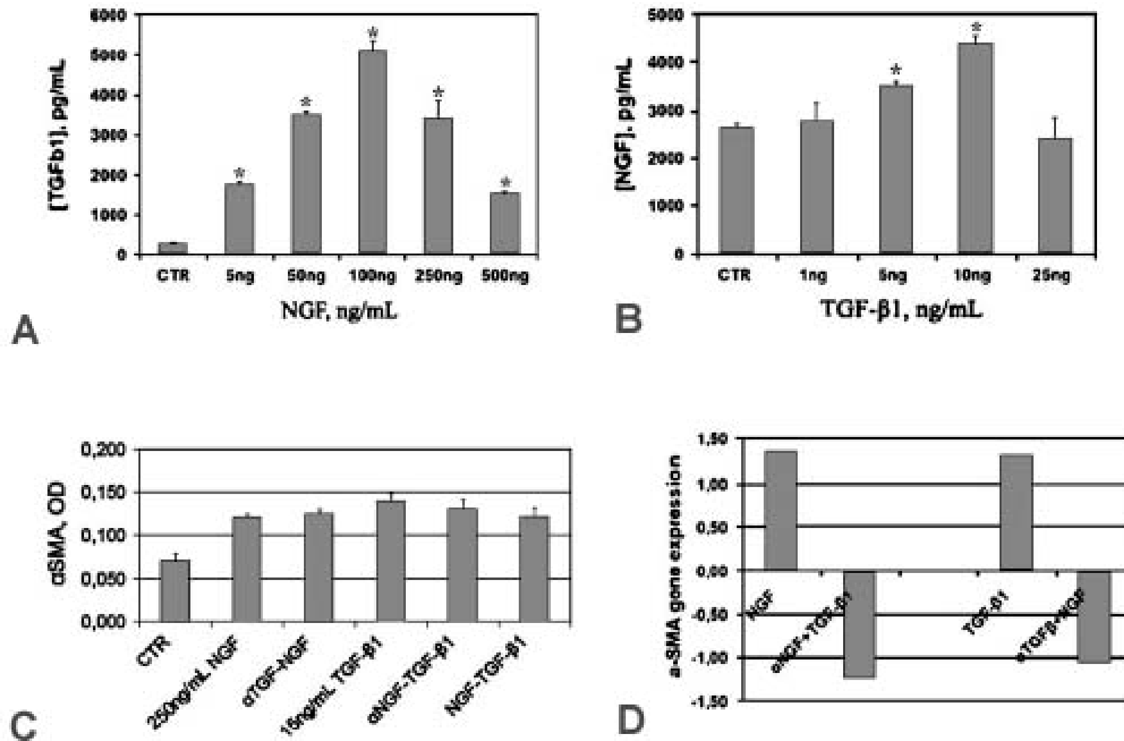

Figura 5.20: Reciproca stimolazione di NGF e TGF-β1, in colture di cheratociti. A,B: analisi ELISA rispettivamente di TGF-β1 e NGF rilasciati dai cheratociti nel medium a seguito dei trattamenti con NGF o TGF-β1. C: test cs-ELISA per αSMA, in cheratociti stimolati con: o 50ng/mL di NGF da solo o insieme all'anticorpo anti-TGF-β1(250ng/mL); o con 15ng/mL di TGF-β1 da solo o insieme all'anticorpo anti-NGF (500ng/mL); o con una mistura di entrambi i fattori: 250ng/mL di NGF e 15ng/mL di TGF-β1). D: dati molecolari di Real-Time PCR, espressi come numero di volte che il trascritto per αSMA, in cheratociti trattati, incrementava rispetto ai livelli basali del trascritto in cellule non trattate.

Capitolo 6

Discussione

In questa tesi di dottorato sono stati studiati gli effetti dell'NGF sui meccanismi di infiammazione e rimodellamento tissutale in due patologie con quadri infiammatori distinti: la Cheratocongiuntivite primaverile (VKC), prevalentemente di tipo Th2 (Th1>Th2) e il Pemfigoide Cicartriziale Oculare (OCP) prevalentemente di tipo Th1 (Th1<Th2).

L'NGF è un fattore pleiotropico, in grado di agire a livello nervoso, endocrino, immunitario e visivo, sia in condizioni fisiologiche che patologiche. L'NGF aumenta durante le malattie infiammatorie, sia acute che croniche, su base allergica o autoimmune, ed è associato ai processi di remodelling tissutale. Diversi studi su modelli animali ed in vitro su cellule di linea e colture primarie attribuiscono all'NGF un ruolo chiave nella modulazione sia dell'infiammazione che dei processi riparativi. I FBs, che rappresentano le cellule target ed effettrici del processo di healing fisiologico e/o patologico, interagendo sia con cellule infiammatorie (neutrofili, macrofagi, linfociti, mastociti ed EOs) che strutturali (epiteliali e endoteliali), sono il tipo cellulare principalmente coinvolto nel processo di remodelling associato alla fibrosi. La loro attivazione, migrazione nel tessuto danneggiato ed il loro differenziamento in myoFBs, sono caratteristiche alla base del processo di riparo e/o di remodelling e la loro sopravvivenza incontrollata sembrerebbe la causa del processo fibrotico. Da studi in vitro è emerso che queste cellule rispondono attivamente a diversi fattori profibrogenici (TGF-β1, PDGF, FGF)

ed in particolare all'NGF. Nella pratica oftalmologica, l'NGF viene usato inoltre come trattamento topico nella cura delle ulcere corneali che normalmente non rispondono alle terapie convenzionali, favorendone la riparazione (processo di healing).

Nella fase iniziale del progetto di tesi sono state utilizzate le biopsie congiuntivali di VKC, OCP e di soggetti sani per sviluppare in coltura cellule primarie di FBs. Uno studio di caratterizzazione fenotipica dei FBs ottenuti da biopsie di VKC ed OCP, in relazione a quelli di controllo, ha evidenziato la presenza di myoFBs (αSMA). Sia nei tessuti di VKC che di OCP, sono state individuate cellule αSMA positive, confermando la presenza di myoFBs nella congiuntiva di questi pazienti. La distribuzione dell'NGF risulta alterata sia nelle biopsie di VKC che in quelle di OCP, a supporto di un coinvolgimento dell'NGF nelle suddette patologie oculari. Mentre nei tessuti di VKC, l'NGF è fortemente incrementato, nei tessuti di OCP invece questo è praticamente assente nell'epitelio e se ne osserva una maggior espressione nello stroma, probabilmente in relazione al tipo di infiammazione e/o di cellule immunitarie/strutturali coinvolte.

In vitro, i FBs/myoFBs derivati da tessuti di VKC producono e rilasciano alte quantità di NGF a cui sono anche recettive esprimendo entrambi i suoi recettori: trkA^{NGFR} e p75^{NTR}. La stimolazione di queste cellule con NGF esogeno non ha effetti sul fenotipo αSMA dei myoFBs mentre si traduce in un aumento nella produzione di MMP9, contribuendo notevolmente al metabolismo della matrice extra-cellulare (ECM). L'espressione della MMP9 è modulata dall'NGF in modo dose-dipendente, con un picco massimo a 10ng/mL NGF sulla sintesi e l'attività della MMP9. La MMP9, svolge un ruolo chiave nel metabolismo della ECM, guidando specificatamente il catabolismo del collagene di tipo IV altamente espresso nei tessuti di VKC. L'NGF potrebbe favorire la migrazione dei FBs verso il sito danneggiato facilitando la digestione della ECM attraverso la modulazione di questo enzima.

I FBs di OCP, invece, mostrano un'associazione tra NGF ed il fenotipo dei myoFBs. I FBs/myoFBs di OCP tendono a sintetizzare e a rilasciare più NGF, probabilmente nel tentativo di recuperare i normali livelli di NGF totale, che nel tessuto congiuntivale di OCP risulta

chiaramente diminuito, e/o per il proprio utilizzo autocrino dal momento che gli stessi FBs esprimono i recettori dell'NGF. Nell'OCP, il fenotipo principale è rappresentato dai myoFBs, in associazione al recettore $p75^{NTR}$. Dall'analisi biochimica dei singoli FBs/myoFBs isolati da biopsie ottenute da pazienti OCP sia allo stadio early-fibrosis (stadio I) che severe-fibrosis (stadio III/IV) è emerso che l'espressione di αSMA e dei recettori $trkA^{NGFR}/p75^{NTR}$ appare strettamente legata allo stadio patologico e/o all'evoluzione della malattia. La percentuale dei myoFBs αSMA e $p75^{NTR}$ positivi aumenta con il progredire della patologia (alto negli stadi avanzati). L'espressione del $p75^{NTR}$ si contrappone a quella del $trkA^{NGFR}$, che è presente nei primi stadi della malattia, in parte co-espresso con $p75^{NTR}$, e diminuisce drasticamente negli stadi avanzati. Il trattamento con NGF stimola l'espressione di $trkA^{NGFR}$ ed inibisce quella di αSMA e del $p75^{NTR}$ nei FBs ottenuti da pazienti ad uno stadio precoce della patologia.

Nei FBs congiuntivali di controllo il $p75^{NTR}$ è poco espresso, se non assente, al contrario del $trkA^{NGFR}$ che è costitutivamente espresso. Sia nella VKC che nell'OCP, i FBs/myoFBs risultano $p75^{NTR}$ positivi, a supporto di un contributo del $p75^{NTR}$ nell'attivazione dei myoFB e nel processo di rimodellamento tissutale e/o di fibrosi. Da studi su altri sistemi, è possibile ipotizzare che $p75^{NTR}$ possa contribuire al processo patologico intervenendo sul fenotipo dei FBs (mediante riorganizzazione del citoscheletro) attraverso l'attivazione del fattore di trascrizione Rho, coinvolto nella regolazione della morfologia dei myoFBs [245]. Questi studi supportano un ruolo protettivo del $p75^{NTR}$ nelle malattie fibrotiche del fegato, dove guida gli epatociti verso la differenziazione in cellule stellate epatiche determinanti nei processi riparo tissutale [246].

Diversamente dai FBs di VKC, quelli di OCP non sono modulati dall'NGF nei processi di sintesi e rilascio di MMP9, suggerendo un ruolo secondario dell'NGF nel metabolismo dell'ECM. Inoltre l'NGF influenza il fenotipo αSMA nello stesso modo di come influenza l'espressione del TGF-β1, suggerendo la possibilità che l'NGF possa agire anche in modo indiretto, sull'espressione $p75^{NTR}\backslash\alpha$SMA, modulando l'espressione del TGF-β1. Da studi mirati è emerso che l'NGF, come il TGF-β1, stimola la migrazione e la differenziazione in

myoFBs, e che la loro reciproca stimolazione non mostra comportamenti sinergici o aditivi sul fenotipo αSMA. La capacità dell'NGF di influenzare l'infiammazione attraverso il rilascio di citochine è stata inoltre studiata nei FBs/myoFBs di OCP. Da un primo studio è emerso che i livelli di IL-2, IL-4 e TGF-β1 sono alti nelle fasi precoci di OCP, dove l'infiammazione è attiva, e diminuiscono in condizioni più severe della patologia. L'esposizione ad NGF risulta in una diminuzione significativa dell'espressione di IL-2, IL-4 e TGF-β1, nei FBs ottenuti da OCP in fase iniziale, mentre determina un drastico aumento l'espressione dell'IL-2, non associata a IL-4 e TGF-β1, in quelli ottenuti da OCP in fase tardiva. Questo studio suggerisce come l'NGF possa agire da una parte sulla sopravvivenza/apoptosi dei linfociti (via IL-2) e dall'altra sui FBs/myoFB modulando la sintesi dei fattori profibrogenici (IL-4 e TGF-β1) a seconda dello stato attivo o non del FBs, contribuendo al fenomeno infiammatorio locale.

Come recentemente ipotizzato in altre patologie, il fenomeno di rimodellamento tissutale mediato dai FBs/myoFBs, potrebbe essere influenzato (modulato/esacerbato) dalla microflora popolante i tessuti (biofilm) e dall'espressione dei TLRs sia in cellule strutturali (FBs) che immunitarie (EOs, mastociti e linfociti Th2).

I TLRs, recettori dell'immunità innata, attivano una serie di risposte che producono mediatori e citochine pro-infiamatore nella prima fase antimicrobica [237]. Come prima barriera di difesa l'epitelio, insieme alle cellule immuno-competenti, sono responsabili della sequela di risposte immunitarie che possono portare sia all'attivazione di altre cellule immunitarie che strutturali come i FBs [247, 237]. Pertanto è stata ipotizzata una possibile risposta dei FBs a stimoli diretti da parte della microflora locale, responsabile di cambiamenti del microambiente, e la possibilità che l'NGF possa intervenire su questo equilibrio immunitario indirizzando la risposta infiammatoria locale. Abbiamo quindi valutato l'espressione dei TLR4 e TLR9, alterati nella VKC [241], sia sui FBs/myoFBs di VKC che OCP [242]. Mentre nei FBs/myoFBs di OCP entrambi i TLRs risultano aumentati in quelli di VKC sono negativamente regolati rispetto i normali controlli. Questo dato potrebbe essere legato ad una diversa storia "immunitaria" delle due malattie. Nell'OCP infatti sembra che i FBs

siano ipersensibilizzati verso la risposta microbica che può contribuire nel mantenere il FBs-myoFbs in uno stato attivo non suscettibile all'apoptosi. L'NGF esogeno sembra modulare questa espressione sia in cellule di VKC che di OCP, forse nel tentativo di riportare i livelli dei due recettori in uno stato di equilibrio.

Da questi studi è emerso che l'effetto pro-fibrogenico dell'NGF potrebbe esser parzialmente dipendente dal rapporto di espressione tra i recettori trkA^{NGFR} e p75^{NTR} sui FBs/myoFBs, dalla relazione con il TGF-β1 e dall'espressione dei TLRs. In particolare nell'OCP è possibile che questi rapporti siano alterati e ciò spiegherebbe il diverso comportamento dei FBs/myoFBs sia ai diversi stimoli dell'NGF che ai diversi stadi della patologia. Il processo di riparo è caratterizzato da diversi steps quali l'eliminazione dello stato patologico, l'apoptosi dei FBsmyoFBs attivati, il rimodellamento dell'ECM, ed in fine il ristabilirsi delle normali funzioni fisiologiche del tessuto. La regolazione temporale e spaziale di questi processi è essenziale nel corretto rimodellamento e recupero del tessuto. Il p75^{NTR} potrebbe svolgere un duplice ruolo: inizialmente essere associato all'attivazione dei myoFBs e poi svolgere un ruolo compensatorio (apoptosi). Riguardo il metabolismo dell'ECM, l'NGF contribuirebbe alla riorganizzazione dell'ECM in modo non specifico visti i diversi effetti sulla MMP9 nei FBs di VKC ed OCP.

In conclusione possiamo riassumere i risultati originali di questa tesi dicendo che l'NGF stimola la migrazione, la differenziazione dei FBs in myoFBs e contribuisce, nella fase finale del processo di healing, alla contrazione dei myoFBs dell'ECM in modo ottimale in FBs non patologici, dove i livelli di TGF-β1 o dell'IL-4 o dei TLRs non sono alterati, suggerendo che il fattore NGF influenza il processo di rimodellamento ma interagendo con una serie di altri fattori ambientali e non.

Capitolo 7

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Mini review

Nerve growth factor and tissue repair remodeling: trkA^{NGFR} and p75^{NTR}, two receptors one fate

Alessandra Micera [a,b], Alessandro Lambiase [c], Barbara Stampachiacchiere [b,c], Stefano Bonini [c], Sergio Bonini [d], Francesca Levi-Schaffer [a,e,*]

[a] *Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Israel*
[b] *IRCCS-G.B. Bietti Eye Foundation, Rome, Italy*
[c] *CIR, Laboratory of Ophthalmology, University Campus Bio-Medico, Rome, Italy*
[d] *Second University of Naples and Institute of Neurobiology and Molecular Medicine, National Research Council, Rome, Italy*
[e] *David R. Bloom Center of the School of Pharmacy, The Hebrew University of Jerusalem, Israel*

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Abstract

This review deals with the role of nerve growth factor (NGF) in healing process as a result of injury. The role of both trkA^{NGFR} and p75^{NTR} specific NGF receptors and their contribution in the complex network of tissue repair process, is discussed and highlighted in view of recent findings. In fact, NGF represents a significant advance in the treatment of etiologically different ulcers (corneal ulcers, pressure ulcers, post-viral infections, chemical burns) and might shorten the recovery process. For these diseases, no specific treatment is actually available. It is reasonable that apart from NGF and/or neurotrophins a different time-course of trkA^{NGRF}/p75^{NTR} expression, might regulate the final process.

In summary, these novel findings on the potential pro-healing capacity of NGF might open new possibilities for this growth factor in modulating the healing processes in several pathological conditions.
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Keywords: NGF; trkA^{NGFR}; p75^{NTR}; Wound-healing; Fibrosis

1. Introduction

Every insult (i.e. chemical, physical, bacterial), that causes acute or chronic inflammatory reactions, can induce tissue damage (injury) that eventually leads to healing. The entire process is a complex mechanism with an interplay of epithelial–stromal–inflammatory cell–cell interactions [1,2]. The rate of healing is influenced by several factors such as location of the lesion, the type of tissue, the entity of eventual infection, poor blood circulation or the eventual use of steroids.

The healing process requires an interaction between several mediators, including cytokines (IL6, IL1β, TNFα) and growth factors (TGFβ1, EGF, PDGF), released by structural and immune or inflammatory cells, in order to achieve a normal recovery of the injured tissue [3,4]. The healing process is generally divided into three interrelated/overlapping phases: (1) inflammation, (2) granulation tissue formation, and (3) tissue remodelling [1,5]. The inflammatory response occurs to remove the debris and necrotic tissue from the injured area and to reduce eventual infection. Haemostasis precedes the inflammation since a fibrin clot (due to platelet) covers the wound and brings wound edges together, serving as a matrix for invading cells and as a reservoir of growth factors and cytokines. Vasodilatation of the existing blood vessels and increased permeability for neutrophils, monocytes (that rapidly differentiate into macrophages) and lymphocytes occur

* Corresponding author at: Department of Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, POB 12065, Jerusalem 91120, Israel. Tel.: +972 2 6757512; fax: +972 2 6758144.
E-mail address: fls@cc.huji.ac.il (F. Levi-Schaffer).

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on the wound site. Neutrophils exert their main role by early phagocytosis of wound debris and release an array of cytokines and growth factors, representing the first signals to activate migration mainly of fibroblasts (FBs) and epithelial cells [6,7]. Later, macrophages digest the fibrin clot, exert antimicrobial functions (phagocytosis and the generation of reactive radicals) and release several growth factors/cytokines that further activate/regulate FBs [4]. Granulation tissue formation comprises FB proliferation, angiogenesis, re-innervations and extra-cellular matrix (ECM) metabolism [2]. Angiogenesis occurs to supply the granulation tissue with fresh nutrients and oxygen through new vessels [1].

FBs differentiate into myofibroblasts (myoFBs), which secrete large quantities of fibronectin, to promote further cell adherence and the suitable condition for the deposition/orientation of the collagen fibrils. Moreover, they produce MMPs and TIMPs involved in the regulation of matrix degradation [7,8]. Once the migrating edges have closed and a collagen-rich matrix has developed, the rate of epithelial cell proliferation falls, myoFBs are down-regulated, collagen production is reduced and the tissue gets back its original morphology and function.

Remodelling of the immature tissue starts simultaneously with granulation tissue formation, but it is often regarded as the third phase of wound healing, where granular tissue changes into a mature scar, which is less cellular and less vascular than the granulation tissue [8,9]. The decrease in cellular component, including myoFBs, might be due to cell migration out of the wound site or to selective apoptosis [10]. The mechanisms that can cause apoptosis, an attractive way to resolve granulation tissue formation, are not clearly understood but oxidative stress, nitric oxide, calcium, proteases, nucleases, mitochondria, growth factors and cytokines are considered crucial mediators [10].

The events of acute wound healing (inflammation, proliferation and remodelling) create a fine-balanced environment in which proteolytic activity and matrix synthesis occur under tight regulation leading to ‘‘regular’’ scar. However this balance is lost in chronic wounds, where absence or recurrence of healing are often observed [11]. Diabetic skin wounds and pressure ulcers are the most common types of chronic wounds. Also repeated allergen exposure in asthmatics, can result in epithelial damage and subsequent uncontrolled/pathological repair such as in hypertrophic scar, keloids and atherosclerosis [12,13]. In addition, chronic wounds, due to repeated insults, usually become heavily colonized with bacteria as a result of hypoxia, poor blood-flow and/or the prolonged period of time that the wound is open, damaging the cells and finally the tissue. Fluids from chronic wounds were found to block in vitro both proliferation and angiogenesis, whereas fluid from acute wounds stimulates in vitro proliferation of FBs, epithelial cells and endothelial cells [11,14].

A ‘‘normal’’ innervation is also required for physiological repair, as observed during diabetic neuropathies, where ulcers do not repair successfully [15]. The reason is unknown and an explanation might be that neuropeptides are able to stimulate both FB and epithelial cell proliferation/activation and modulate local blood flow, neurogenic inflammation and immune response [16]. Nerve regeneration takes place together with angiogenesis, during granulation tissue formation [1].

In summary, the wound-healing process can be physiologically balanced or can result in an overt pathological process (scar and fibrosis) [1,2]. Growth factors play a crucial role in driving wound-healing process as supported by their local increase [4,6]. Therefore, it appears that NGF, and likewise its specific receptors, found increased in all fluids and tissues from several injured tissues [17,18], might be important factors contributing to the physiological repair [19,20].

2. NGF: brief history and function at glance

NGF, discovered by Rita Levi-Montalcini in collaboration with Stanley Cohen,was originally characterized by its ability to stimulate growth, differentiation, survival and maintenance of peripheral sensory and sympathetic neurons during development and after injury [21]. NGF represents the archeotype and most probably the best characterized member of a structurally and functionally related family of neurotrophins (14 kD/240–260 aminoacids), including brain derived neurotrophic factor (BDNF) and neurotrophins (NTs) 3, 4/5 and 6 [22]. A highly conserved molecule among different species, NGF is a glycoprotein of 118 aminoacids, consisting of three sub-units (α_2, β, γ_2; molecular weight 130 kD), whose gene lays in the proximal short arm of chromosome 1 [22]. The β-NGF is responsible for the NGF biological activity, the γ-NGF is a highly specific active protease, that is able to process NGF precursor to its mature form, and the α-NGF appears inactive [22].

Like the other NTs, NGF is produced in the nervous system by accessory cells, such as astrocytes and oligodendrocytes, which in turn regulate the differentiation and survival process of neurons mostly through the formation of paracrine circuits [21]. NGF was found to be produced and utilized by several non-nervous cell types, including immune inflammatory cells, epithelial cells, keratocytes, and smooth muscle cells, indicating that NGF may have roles outside the nervous system [23–25]. Because of its activities outside the nervous system, NGF is actually recognized as a pleiotropic factor.

NGF accumulates in different tissues and in the peripheral blood, but the mechanisms regulating NGF synthesis and release have not yet been completely clarified [21]. In the tissues, NGF is synthesized as a precursor (proNGF) and undergoes post-translational processing (intracellular cleavage by both the serine protease plasmin and the matrix metalloproteinase MMP7 [26]) to generate and secrete the mature β-NGF form [22–27]. While submandibular gland

releases mature NGF, in the other tissues (prostate, hair follicles, thyroid gland, retina, skin, colon, dorsal root ganglia as well as central nervous system) NGF is mainly secreted as proNGF or at least a mixture of both forms [22,27]. The fact that neither proNGF nor intermediates have been found in the saliva, implies that cleavage/processing in the submandibular gland occurs before secretion, as confirmed by specific cell expression studies [27]. Chemically, proNGF is more long-lived, less basic and shows an isoelectric point different from those of mature NGF (IP_{proNGF} 8.1 versus IP_{NGF} 9.3) [27].

Although proNTs have been considered for a long-time as inactive precursors, recently they have been found to exert biological activities, even stronger than those of related mature forms [27]. In fact, the specific activity of proNGF in stimulating neurite outgrowth (based on EC50) is almost five-fold less than that of mature NGF, indicating that proNGF might exert neurotrophic functions as well [27]. By the way, most of the proNGF functions encompass apoptotic effects [21–27], most probably by re-organization into higher order structures for selective binding to $p75^{NTR}$ [27]. Besides some molecular and biochemical information, the function of these precursors and their intermediates is still poorly understood.

2.1. NGF and chronic inflammatory-allergic response

The first evidence of NGF association with allergic disorders occurred in 1995 when our group [28] reported increased NGF plasma levels in patients with vernal keratoconjunctivitis (VKC), an allergic (Th2 driven) ocular condition, showing extensive tissue remodeling [29]. Surprisingly, NGF expression correlated with the increased total IgE levels, a common peripheral marker of allergy, and with the increased number of recruited mast cells (MCs) and infiltrating eosinophils (EOs) in the tarsal and bulbar conjunctiva of patients having the active form of the disease [28,29]. This correlation implied a link between NGF, probably originated from tissue MCs and likewise EOs [30–32], and the allergic ocular response.

The discovery of NGF activity on MCs, key cells of the allergic response and important cells in parasite infections, occurred in 1977 when Aloe described the role of NGF in the growth and survival of newborn rat MCs, as well as their reduced number in Schistosoma mansoni-infected rats, in the presence of neutralizing anti-NGF antibodies [32,33]. Later on, NGF was shown to induce MC development from mouse bone marrow cells in the presence of IL-3, to promote human and leukemia cell line MC maturation by inducing chymase expression, and to increase rat peritoneal MC survival by inhibiting in vitro their apoptosis [34].

The observation of the increased NGF plasma levels in VKC prompted the investigation of the presence of NGF in other allergic diseases. In 1996, our group detected a significant increase of circulating NGF in patients suffering from allergic asthma, urticaria-angioedema and allergic rhinoconjunctivitis [35]. Increased NGF levels were also found in nasal and bronchial alveolar lavage fluids obtained from allergic patients [36]. There is now a substantial body of evidence demonstrating the involvement of NGF in the allergic reaction, in terms of EOs and lymphocyte infiltration, MC recruitment as well as cytokine release (IL-4, IL-5, IL-10, etc.), plasma cell differentiation and IgE production/secretion [25,37].

In spite of all these observations, how NGF participates in the allergic inflammation and which cells – other than MCs and EOs – are responsible for NGF increase in these conditions, are actually unknown. Since in allergic inflammation and asthma NGF might be either pro- or anti-inflammatory [25,36], the role of NGF is still controversial. In addition, the fact that in the airways epithelial cells, submucosal glands and stromal cells (fibroblasts) actively produce and utilize NGF during allergic response, would suggest a "physiological" reparative role of NGF in addition to the inflammatory one [19,20].

2.2. NGF and healing process: a lesson from experimental and therapeutic approaches

Although the best documented activity of NGF is on nerves, there is evidence that NGF also plays part in repair process. The observation that NGF exerts a "role" in wound-healing is not new. Earlier studies showed that removal of submandibular gland in mice retarded the rate of contraction of experimentally induced wounds [38]. Next, Li and colleagues in 1980 showed that topically applied NGF accelerated the rate of wound contraction in sialoadenectomized animals, suggesting that in mice the reflex of wound-licking might serve to introduce/accumulate NGF as well as other healing accelerators to promote cutaneous healing [39]. Five-year later, this hypothesis was confirmed by Lawmann et al. [40], who showed that NGF was effective also in improving outgrowth of sciatic nerve and in stimulating proper healing after muscle contusion, fracture or even vasculitis [40–42]. The pharmacological effect of NGF in triggering wound healing was also observed in both normal and healing-impaired diabetic mice [43]. This last finding was of great importance in indicating NGF as a possible agent of healing in human disorders.

In humans, the evidence of NGF effect in priming healing occurred when in an open-study, our group observed that NGF eye-drop restored corneal integrity in patients having chronic corneal neurotrophic ulcers [44,45]. This observation was investigated at the structural, biochemical and molecular level in a rat model of corneal injury, suggesting a multi-functional action of NGF on epithelial cells [46]. Significant clinical results were also obtained in the topical NGF treatment of severe chronic skin ulcers (pressure or vasculitis ulcers), indicating that NGF might be an effective therapy in ulcers hard to treat or orphan of any conventional therapy [42,47,48].

3. NGF and two structurally unrelated transmembrane receptors

Some growth factors display two distinct surface receptors, which collaborate in mediating the signalling [26]. The presence of two distinct receptors with specific/independent cellular activities represents a "unique" property for NGF in comparison to the other growth factors and cytokines [22]. In fact, both receptors bind to NGF either alone or together, instead of working at different and subsequent steps, such as TGFβ-RI and TGFβ-RII [49].

NGF binds independently to the tropomyosin trkA tyrosine kinase receptor (termed p140trkA^{NGFR} or simply trkA^{NGFR}) and the p75 pan-neurotrophin receptor (named p75^{NTR}) [22,50,51]. While p75^{NTR} binds all NTs with equal affinity (1 nM Kd), trkA^{NGFR} is very specific in its binding (10 pM Kd). In the presence of trkA^{NGFR}, p75^{NTR} can participate in the formation of high-affinity binding sites, resulting in enhanced NGF responsiveness, as well as selective binding, in which duration and magnitude depend on the ratio of p75^{NTR} and trkA^{NGFR} on cell surface (Fig. 1) [52,53]. As recently reported, p75^{NTR} contributes to the selective trkA^{NGFR} recognition of NGF, since in the absence of p75^{NTR}, a trkA^{NGFR}-BDNF specific binding occurs in vitro [22,54]. Upon binding and following internalization, NGF triggers a cascade of biochemical events, in which signal transduction pathway has been extensively investigated in the pheochromocytoma 12 (PC12) cell model [52,53].

3.1. The tropomyosin receptor trkA^{NGFR}: a more complex signalling cascade

trkA^{NGFR} is a transmembrane glycoprotein belonging to the family of receptor tyrosine kinase, called trks (trkA^{NGFR}, trkB^{BDNFR}, trkC$^{NT3/5R}$), encoded by a gene located on chromosome 1 [22]. The discovery of the trkA^{NGFR} form was several years later than those of p75^{NTR} [21]. However, it became more attractive due to the tyrosine kinase features and the complex network of signaling displayed [22,51]. The cascade includes the MAPK-Ras-Erk pathway, phospholipase Cγ1, P3I kinase and SNT proteins (suc-associated neurotrophic factor-induced tyrosine-phosphorylated target) [22,55]. Interestingly, the activation of the Smad signal transduction pathway, a typical pathway used by members of the TGFβ family, has been also reported in NGF treated PC12 cells [56].

Most of the NGF-mediated biological activities (from differentiation/activation to proliferation/survival) are due to ligand-dependent trkA^{NGFR} auto-phosphorylation and subsequent activation of several signal transduction cascades. TrkA^{NGFR}-mediated cell rescue involves either the activation of survival signals or the suppression of death signals

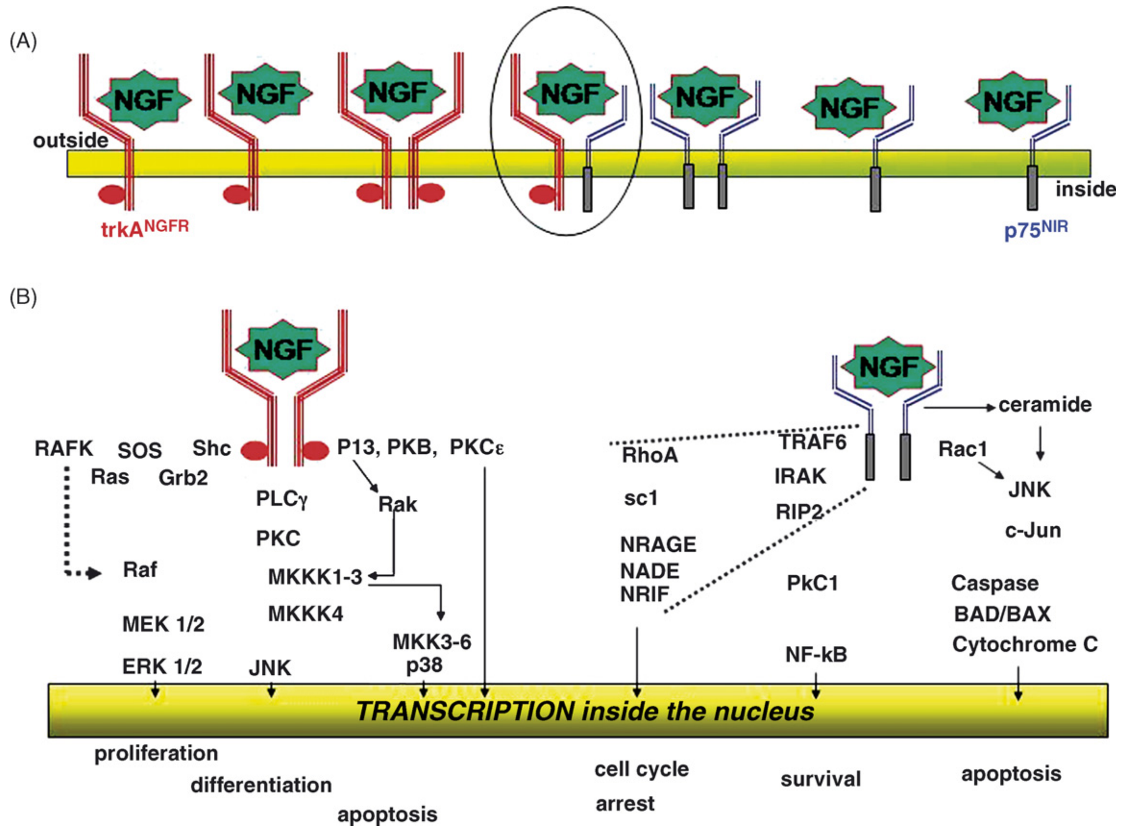

Fig. 1. A schematic overview of both trkA^{NGFR} and p75^{NTR} signal transduction pathways. (A) trkA^{NGFR}-p75^{NTR} dynamic expression on the cell surface. (B) Flow-chart of trkA^{NGFR} (left panel) and p75^{NTR} (right panel) signaling.

mediated by p75^{NTR} [52]. Interestingly, trkA^{NGFR} has been recently reported to cause apoptosis, in trkA^{NGFR}-transfected (40,000 of each receptor/cell (p75^{NTR}/trkA^{NGFR})) PC12 in comparison to parental ones (40,000 p75^{NTR}/cell and 400 trkA^{NGFR}/cell) PC12 [57]. In particular, in PC12 cells with up-regulated trkA^{NGFR}, NGF-induced cell death was merely trkA^{NGFR}-mediated, rather than p75^{NTR}. These opposing NGF effects, ranging from survival to cell death, are apparently not shared by the other NTs, although all bind p75^{NTR} [57].

3.2. The pan-neurotrophin receptor p75^{NTR}: a still obscure signaling pathway

Compared to trkA^{NGFR}, the functional role that p75^{NTR} plays in NGF signaling is more complex and less clearly defined [26,53]. p75^{NTR} mostly correlated with development, was initially proposed to play a role as a ‘‘surface reservoir’’ for NTs on glial cells, presenting NTs to ingrown axons [58], or as a ‘‘cleaner’’ for excess NTs encountered by neurons in target tissues [59]. Later, p75^{NTR} was found to play a role as a ‘‘helpful’’ receptor for trks, either enhancing trk responsiveness in the case of NGF and trkA^{NGFR} or increasing ligand-specificity in the case of BDNF or NT3 and trkA^{NGFR} [54,60].

p75^{NTR} is a 399 aminoacids type-I transmembrane protein, whose extra-cellular domain contains four repeated modules of six cysteines, a typical structure of the TNFR superfamily (Fas/TNF receptor superfamily), and devoid of intrinsic catalytic activity [22]. Recently, p75^{NTR} has been accepted as a trk-independent signaling receptor, even though specific transduction signal has not been completely elucidated as yet. In fact, p75^{NTR} signaling involves activation of NF-kB (Rel/NF-kB transcription factors) and the phosphorylation of the transcription factor c-Jun kinase (JNK), as well as increased production of ceramide, leading to gene transcription or programmed cell death [51].

p75^{NTR} is merely a receptor of development, as observed in the CNS, and it increases during injury or diseases [61]. It is noteworthy that, in view of recent findings on tumors, p75^{NTR} has been proposed as a prognostic marker [62]. p75^{NTR} functional role is contradictory being both pro-survival and pro-apoptotic [26,53,63]. Other studies showed that NGF mitogenic effect required trkA^{NGFR}, but anti-apoptotic effect of NGF was mediated by p75^{NTR} throughout NFKB pathway [64].

An innovative finding came from the discovery of a 94 kDa-cytoplasmic protein known to interact with p75^{NTR}, named neurotrophin receptor-interacting factor (NRIF), which associates with the cytoplasmic domain of p75^{NTR} and is possibly released upon NGF specific binding [53]. As mentioned above, proNGF is a high-affinity ligand for p75^{NTR}, inducing p75^{NTR}-dependent apoptosis in cultured neurons with minimal activation of trkA^{NGFR}-mediated differentiation or survival [26,27]. This observation strengthens the premise that the biological activity of NTs is regulated by proteolytic cleavage, with proforms preferentially activating p75^{NTR} to mediate apoptosis, and mature forms activating trkA^{NGFR} receptors to promote survival [53].

3.3. Cell activities in relation to trkA^{NGFR} and p75^{NTR} selective expression

NGF-triggered cellular activities comprise: migration, proliferation, differentiation, survival and even apoptosis. These opposing effects, not common to the other NTs, are most probably related to the ‘‘exclusive’’ properties of NGF receptors.

The contribution of NGF in both innate and adaptive responses is actually well documented [25,37]. Historically, the association of NGF with the immune system arises from the discovery that MCs, involved in native immunity, wound healing as well as in the maintenance of tissue homeostasis/repair and recognized as a pivotal cell in allergy [65], are able to synthesize, store, release and autocrinally utilize NGF, via trkA^{NGFR} expression [33,34]. In vitro, NGF can replace totally or partially stem cell factor (SCF) which is the key growth factor responsible for MC differentiation, survival and activation [34]. In addition, NGF induces the development of MCs from mouse bone marrow cells in synergy with IL-3 [33]; NGF promotes maturation/phenotypic changes of human cord blood-derived MCs, as evaluated by the expression of the α/β tryptase and chymase [34]; and NGF increases the survival of MCs from rat peritoneal cavity, by suppressing their apoptotic pathway [33,34]. Most of the observed effects are mediated by trkA^{NGFR}.

Although NGF is a neurotrophin mandatory for the development and function of peripheral and central neurons, playing a crucial role during nerve injury [21], old and recent findings have shown that NGF regulates immune and inflammatory responses through direct and/or indirect effects on immune-competent cells [66]. Particularly, NGF enhances IL-2 receptor expression on human natural killer (NK) cells, indicating an NGF role in the innate immune response [66]. NGF triggers the differentiation from human peripheral blood [70], suppresses apoptosis and enhances functional properties of monocytes/macrophages and neutrophils, via trkA^{NGFR} [31,32,67]. In specific apoptosis studies, p75^{NTR} appeared to play a minimal role in Fas-mediated neutrophil apoptosis [31]. Conflicting data exist on their trkA^{NGFR}/p75^{NTR} expression; while peritoneal macrophages express both receptors, human macrophages, populating the palatine tonsil, thymus, optic nerve, and retina do not express p75^{NTR} [68]. Specifically, infiltrating monocytes produce and utilize NGF via trkA^{NGFR}, while p75^{NTR} appears mainly on macrophages during the inflammatory states, as viral infections [69]. Even though most of the NGF effects were found to be mediated by trkA^{NGFR}, p75^{NTR} has been recently described in human macrophages [69]. In vitro, NGF promotes granulocyte differentiation from hematopoietic stem cells [70]. NGF

suppresses apoptosis and enhances functional activities in both neutrophils and eosinophils [71,72].

Chronic inflammatory and allergic states are associated with a consistent infiltration, activation and survival of EOs [73]. Comparable to MCs, NGF induces maturation of EOs from progenitors and affects both in vitro migration/adherence, survival and release of cytokines/mediators, most probably trkA^{NGFR} mediated both under physiological and pathological conditions [20,32]. No evidence indicates the presence of p75^{NTR} on these cells.

NGF acts as a neuroimmune signal playing a role in the development and organization of the spleen as well as in the organogenesis of thymus [74]. TrkA^{NGFR} and p75^{NTR} are expressed on myeloid and lymphoid tissue-cells and NGF is able to modify, in a dose-dependent manner, cell proliferation, differentiation, survival and functions [37].

The observation of a strong expression of NGF and trkA^{NGFR} in developing and adult lymphoid organs, suggests a trkA^{NGFR} mediated NGF control of early hematopoiesis and immune cell differentiation [74]. Recently it was found that, NGF pathway is regulated by toll-like receptor-2 (TLR2), one of the best-characterised receptors of the innate response. TLR2 appears responsible for p75^{NTR} up-regulation during bacterial infections, as observed in cultured splenocytes, providing additional information on the role of NGF in innate reaction [75]. In trkA$^{NGFR-/-}$ mice, thymocyte density was found lower in developing thymus, indicating a role of NGF in organogenesis [76]. Consistently, genetically manipulated animals (displaying p75^{NTR} and non-functional trkA^{NGFR}), showed structural changes consisting of a decrease in the density of thymocytes, and general structural abnormalities. Taken together, these findings suggest the existence of a specific trkA^{NGFR}-NGF pathway in different thymic functions as well as a specific role of trkA^{NGFR} in the development of the murine thymus, while the p75^{NTR} function remains poorly understood.

B lymphocytes bind in a paracrine/autocrine way NGF via both trkA^{NGFR} and p75^{NTR}. This induces B-cell tissue infiltration/proliferation, differentiation into plasma cell (Ig-G A M and Ig-E,), and specific cytokine release (IL-4 and IL-5) [25,37,77]. The survival of B-cells as well as the induction of more specialized memory cells ([IgG/IgA]$^{+}$B cells), is via trkA^{NGFR} [78]. Interestingly, p75^{NTR} shares structural homology with the B cell antigen, CD40, tumor necrosis factor (TNF) receptor and Fas antigen (APO-1), which play a role in cell apoptosis [37].

Most of the studies indicate that NGF modulates T lymphocyte proliferation and differentiation to more specialized immune cells, in a trkA^{NGFR} dependent autocrine manner [77,79]. NGF also induces T-cell dependent antibody synthesis [80], IL-2 receptor expression [66], and finally c-fos gene transcription [37]. The induction of NGF effects, via trkA^{NGFR}/p75^{NTR}, in T-cells appears more complex. Th polarization towards a Th2 phenotype appears to be associated with both NGF production and selective trkA^{NGFR}/p75^{NTR} expression [79]. Consistent with this, Villoslada et al. reported that NGF might exert regulation of Th1/Th2 balance, suggesting a possible modulation of Th1/Th2 expression during pathological conditions, even though the specific receptor was not investigated [81]. Conflicting data encompass receptor expression in resting or activated T-cells: while trkA^{NGFR} was limited to activated cells [82], our group demonstrated the constitutive expression of trkA^{NGFR} in resting T-cells, implying a NGF effect as well during physiological conditions [79].

NGF is produced and utilized by many structural cells including FBs [83], keratinocytes [84] and endothelial cells [85]. This would imply that increased NGF levels might regulate the healing of skin wounds or drive tissue remodeling during chronic inflammatory disorders, by means of NGF multifunctional activities [23]. Heterogenic FBs (skin, lung, conjunctiva and cornea derived FBs) and epithelial cells (including airway, cornea and conjunctiva derived epithelial cells and keratinocytes) produce, release and utilize NGF, mainly via trkA^{NGFR} [23,24,84]. Interestingly, ‘‘quiescent’’ skin, lung and cornea (keratocytes) FBs have been found to express trkA^{NGFR} but not p75^{NTR} [83,86,87]. Control and inflamed conjunctival FBs as well as corneal FBs, express both trkA^{NGFR}/p75^{NTR} [86,87]. All these findings clearly define p75^{NTR} as an ‘‘inducible’’ receptor, typical of activated myoFBs, and open new perspectives to the study of NGF in the healing process. Biologically active NGF is synthesised and released by proliferating human keratinocytes [88]. At the basal epithelial layer, trkA^{NGFR} and p75^{NTR} are expressed by keratinocytes, suggesting a possible autocrine NGF utilization, as well as a possible contribution to either ‘‘normal’’ scar or uncontrolled hyper-proliferative conditions. Moreover, goblet cells of conjunctival and airway root, as well as gland cells, produce and release NGF and express trkA^{NGFR}.

NGF is also an activator of endothelial cells and plays a role in mediating angiogenesis during the healing process. Besides the expression of both receptors, specific trkA^{NGFR}-NGF binding can activate endothelial cell migration and signaling that underlie angiogenic processes [85].

The observation that both keratinocytes, FBs and endothelial cells produce and utilize NGF, and that in turn NGF is effective in accelerating the rate of healing, facilitating tissue repair and angiogenesis, suggests the hypothesis that NGF might be a pluripotent factor for healing tissues. A specific NGF cross-talk might exist between keratinocytes, FBs and endothelial cells, during the complex events of healing, in addition to the other well known growth factors and cytokines.

4. NGF and the proper resolution of tissue repair: two receptors for a proper wound-healing and tissue remodeling process

Cytokines and growth factors exert a crucial role in driving the healing process and their synergistic effects

might underlie a successful wound healing [4,6]. At present, NGF is recognized as a cross-talking factor between all the cells involved in the healing process. After injury, NGF and trkA^{NGFR}/p75^{NTR} are differentially expressed during the healing process and tissue repair [18,61]. The healing activity of NGF, observed in clinical studies [44,45,47,48], was analyzed at the cellular level in several experimental animal and in vitro models [46]. During wound healing and tissue remodeling NGF might influence epithelization, contraction or connective tissue deposition. According to this hypothesis, NGF contribution might be hypothesized to be direct, by priming structural (FBs/myoFBs, epithelial and endothelial cells) or immune resident/infiltrating cells (neutrophils, monocytes/macrophages, lymphocytes, MCs and EOs), or indirect, via the stimulation of other pro-fibrogenic factors [17,83].

Human keratinocytes synthesize and secrete biologically active NGF in a growth-regulated fashion and express trkA^{NGFR} and p75^{NTR}, whose specific binding modulate either proliferation or differentiation [84]. The binding parallels with the course of NGF synthesis and p75^{NTR} expression decrease when a confluent, differentiated and fully stratified epithelium appears [84]. At least in culture, the migration as well as entering the S-phase of the cell cycle of epithelial cells appears to be trkA^{NGFR} mediated [84]. These data provide evidence that NGF, in addition to its effect as a survival and differentiation factor is a potent regulator of migration and cell proliferation in human epithelial cells. The mitogenic effect was either indirect, maybe via EGF production, or direct via integrin upregulation [84].

FBs, previously recognized merely as tissue-scaffold providing cells, are regulatory cells exerting active roles in tissue homeostasis, by producing cytokines and growth factors with autocrine/paracrine activities [7]. FBs influence the function and survival of MCs and EOs, all populating the injured area principally through SCF and GM-CSF, respectively [89]. FBs and myoFBs (also known as granulation tissue FBs) are the principal target/effector cells involved in the stromal repair [7]. During the last stages of healing, an unbalance in the mechanisms regulating FB differentiation into myoFB as well as prolonged myoFB survival and function lead to pathological tissue remodelling and fibrosis [7]. Emerging data suggest an important role of NGF in promoting/accelerating wound healing and tissue repair/remodeling, together with other well-documented pro-fibrogenic factors [9,10]. Compelling in vitro and in vivo evidence shows that FBs are one of the sources/targets of NGF, at the healing sites [18,23,61,83]. Fibrotic human skin, lung or conjunctival tissues display immunoreactivity for NGF, trkA^{NGFR} and p75^{NTR} [83,90,91].

FBs represent the main target and effector cells of healing/repair processes due to their ability to migrate to the injured area, proliferate, produce ECM, differentiate into myoFBs and finally contract the wound [83,86,87]. Compelling old and recent evidence shows that FBs are one of the sources/targets of NGF, both in vivo and in vitro [83,86,87]. Heterogenic FBs differentially express trkA^{NGFR}/p75^{NTR} [11,86]. In particular, these FBs express constitutively trkA^{NGFR}, whereas p75^{NTR} was expressed only after prolonged NGF or specific TGFβ1 exposure, and therefore identified as an inducible form [11,83,86,87]. This unusual aspect has been observed in cell lines of human skin and lung as well as in primary cultures of human conjunctival and corneal FBs [83,86,87]. NGF significantly triggers skin, lung and conjunctival FB migration in a model of wounded FB or in Boyden chambers. Cellular migration is innermost to a wide range of biological and pathological processes, tissue healing included [5]. Therefore, the fact that NGF induced migration of ‘‘wounded’’ lung, skin and conjunctival FBs, as previously observed for keratinocytes, provide additional data to the NGF contribution in the early stages of healing [5]. Interestingly, this effect was trkA^{NGFR} mediated in both cell types, as confirmed by specific neutralizing anti-receptor studies [83,86,84]. Neutrophils, macrophages, lymphocytes as well as MCs and EOs actively release NGF in the injured site, and NGF might be in turn utilized either autocrinally or paracrinally. At the wound site, NGF release is found not to be restricted to inflammatory/immune cells since myoFBs and epithelial cells are able to synthesize and release the factor [18].

Tissue repair is a dynamic process involving not only early inflammation but also granulation tissue formation and remodeling of ECM [1,5]. Granulation tissue formation and contraction of the wound are characterized by the activation of FBs and the appearance of functional myoFBs [7]. MyoFBs contract microfilament bundles that are attached to ECM throughout integrins, resulting in a local matrix contraction [7]. MyoFBs are defined as α-smooth muscle actin (α-SMA) expressing cells, populating the fibrotic tissue [7]. Hasan et al. described for the first time the coordinate expression of NGF and α-SMA in skin wounds of young and adult rats, suggesting an NGF effect on these specialized cells [90]. In 2001, our group extended previous data showing the ability of NGF to trigger the differentiation of skin and lung cell lines of FBs into α-SMA expressing myoFBs [83]. On the other hand, NGF did not induce proliferation of either these cells or of epithelial cells or of keratinocytes [83,84]. These findings were later confirmed on primary cultures of conjunctival and corneal FBs [86,87]. Moreover, NGF did not influence either collagen production, or MMPs production or activation (metabolism of ECM), in skin, lung, conjunctival or corneal FB [24,83,86,87]. Selective NGF effect on FB migration and contraction was not confirmed by Kohyama et al., whose contrasting data are probably attributable to different culture conditions [92]. Interestingly, NGF stimulated TGFβ1 release in conjunctival FBs and *vice-versa* [93,94]. This data is very important since TGFβ1 is the main profibrogenic factor, playing crucial

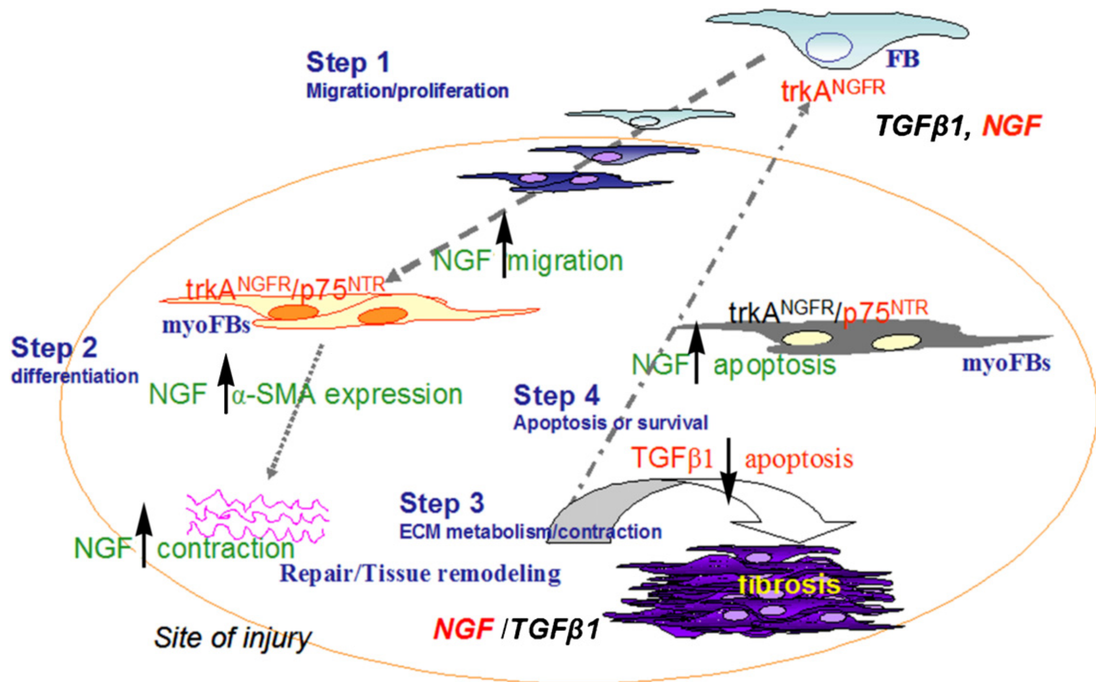

Fig. 2. Model of NGF and fibroblast/myofibroblast cross-talk during tissue repair. A glimpse to NGF receptors. In green are shown the in-vitro observed NGF effects. Quiescent fibroblasts (FBs) express trkA^{NGFR} while myoFBs express both trkA^{NGFR} and p75^{NTR}, and all produce NGF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

roles in tissue remodeling via the smad pathway activation [49,95].

In this complex process, NGF and trkA^{NGFR}/p75^{NTR} were found differentially expressed and this observation might, at least in part, explain some of the selective NGF effects described below [61].

During normal wound healing, as the mechanical tensions decrease, myoFBs disappear mainly via apoptosis [7,9]. If myoFBs persist in the wound, then fibrosis takes over [8]. Specifically in vitro in TGFβ1-induced myoFB experiments (an in vitro model of myoFB cultures [96]), NGF modulated trkA^{NGFR} and p75^{NTR} expression; it increased p75mRNA expression; and finally, it modulated myoFB survival/apoptosis. NGF triggered apoptosis of in vitro induced myoFBs and likewise down-regulated TGFβ1mRNA expression in the myoFBs [Micera et al., unpublished observations]. This aspect of p75^{NGFR} had been previously investigated in other cell types, mainly neuronal and immune cells. The fact that NGF might contribute at least in part to apoptosis of myoFBs, might be of great value in terms of physiological tissue repair (Fig. 2).

Taken together, all these data illustrate a direct pro-fibrogenic effect of NGF on skin and lung FBs and therefore point to more specific roles for NGF in tissue repair and fibrosis.

In relation to TGFβ1, NGF might be a synergistic factor during the early stages and a counteracting factor during the late phases of the process [97]. This latter hypothesis should be proven by additional studies of silencing at the transcriptional level.

5. Concluding remarks and new perspective

The response to injury is characterized by the early inflammatory response, granulation tissue formation and finally tissue remodeling to obtain a fine balanced repair [1,4,5]. Numerous cell types take part in the process by releasing several mediators/factors acting in concert to achieve repair [2,3]. NGF was found to be useful in the treatment of etiologically different ulcers and may shorten the recovery process [44,45]. While the NGF effects on inflammatory/immune cells have been extensively studied, only recently it was observed that NGF is a strong inducer of epithelial cell and FB properties, the effector/target cells of repair process [23]. It is reasonable that apart from NGF, other NTs and likewise a different time-course of trkA^{NGRF}/p75^{NTR} expression might regulate the final healing process. The old and recent findings highlight trkA^{NGFR} and p75^{NTR} as crucial tools in this process, opening new therapeutic strategies. In particular, p75^{NTR} appears as an old receptor with new properties.

Because of the lack of effective pharmacological agents for a well balanced tissue repair, NGF, and likewise the modulation of its receptors, appear as an original and valid therapeutic tool.

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Dr. Alessandra Micera, MSc, PhD student (born 1969) is working in the Laboratory of Ophthalmology, IRCCS-GB Bietti Eye Foundation and Campus Bio-Medico (Rome). She graduated in Biology (1993) at the University ‘‘La Sapienza’’ (Rome), working in the laboratory of Professor Rita Levi-Montalcini (Institute of Neurobiology, CNR, Rome), under the vision of Dr. Luigi Aloe, studying the role of nerve growth factor in autoimmune and neurodegenerative disorders. She improved her knowledge in Molecular Biology (1994–1995) at the Medical Research Council, Cambridge (UK), under the supervision of Dr. M. Goedert. Co-author of 45 papers, published on peer-review international journals, actually she is waiting for PhD-degree in Immuno-Pharmacology (Hebrew University of Jerusalem, School of Pharmacy, Hadassah Medical School, Israel, supervisor Prof. Francesca Levi-Schaffer). Her major field of interest is to study the role of NGF in chronic allergic inflammatory disorders and related tissue remodelling.

Dr. Alessandro Lambiase, MD, PhD (born 1966) is currently an Assistant Professor in the Department of Ophthalmology at the University of Rome ‘‘Campus Bio-Medico’’. He obtained his degree in Medicine (1991) and in Ophthalmology (1996) at the University of Rome ‘‘Tor Vergata’’. He has worked in the laboratory of Professor Rita Levi-Montalcini (Institute of Neurobiology and Molecular Medicine, CNR, Rome, Italy). He was fellow at the Department of Ophthalmology (Ospedale Civile di Venezia) on corneal diseases. He is Author of more than 90 papers. His main field of interest is the study of the pathogenesis and treatment of the ocular surface diseases. In particular the conjunctival allergic inflammation, the treatment of neurotrophic corneal ulcers with nerve growth factor, and the application of corneal stem cells in limbal deficiency.

Dr. Barbara Stampachiacchiere, MSc, PhD student (born 1972) is actually working in the Laboratory of Ophthalmology, IRCCS-GB Bietti Eye Foundation and Campus Bio-Medico (Rome). She graduated in Biology at the University ‘‘La Sapienza’’ (Rome) in 2002, working at the Molecular Biology laboratory (supervisor Dr. Lucia Fabiani). She has worked in the laboratory of Professor Rita Levi-Montacini (Institute of Neurobiology and Molecular Medicine, CNR, Rome), under the supervision of Dr. Luigi Aloe, studying the role of nerve growth factor (NGF) in autoimmune and neurodegenerative disorders. She improved her knowledge in molecular biology at Dermatology Department, University Hospital of Hamburg (short-time/2003), under the supervision of Professor Ralph Paus. Co-author of 10 papers, published on peer-review international journals, actually she is attempting her PhD-program in Medical Microbiology and Immunology at the University ‘‘Tor Vergata’’, under the supervision of Prof. Stefano Bonini (Campus Bio-Medico, Rome). Her major field of interest is to study the role of NGF in chronic autoimmune disorders of the ocular surface and related tissue remodelling.

Prof. Stefano Bonini, MD (born 1953) is the Professor and Chairman of the Department of Ophthalmology at the University of Rome ‘‘Campus Bio-Medico’’. He graduated in Medicine at the University of Rome in 1977. He obtained his degree in Ophthalmology (1981) and Allergy and Clinical Immunology (1984) at the University of Rome La Sapienza. From 1985–1987 he was trained by Dr. Mathea R. Allansmith in Ocular Allergy at Harvard Medical School and Schepens Eye Research Institute in Boston, USA. From 1985–2002 he was part (as assistant professor and associate professor) of the Faculty at the University of Rome ‘‘Tor Vergata’’ and Scientific Director of the G.B. Bietti Eye Foundation in Rome. In 2002 he was appointed Chairman of the Eye Clinic at the University of Rome Campus Bio-Medico. He is the Author of 210 papers and his main field of interest is ocular allergy and the inflammation of the ocular surface. Among his most original papers are the detection of the late-phase allergic reaction in the conjunctiva, the treatment of neurotrophic corneal ulcers with nerve growth factor, and the description of the conjunctiva and cornea stem cells and their clinical transplantation in severe cornea alkali burns.

Sergio Bonini (July 22, 1947) is Professor of Internal Medicine at the Second University of Naples, Italy. Research areas of Prof. Bonini include allergic and autoimmune diseases with studies on autoantibodies, IgE, allergens, mechanisms of allergic inflammation, genetics of atopy. Recent research has mainly dealt with allergic eye disease as a model of allergic and eosinophilic inflammation. Current studies are focused on neural (nerve growth factor) and endocrine (sex hormones) modulation of allergic inflammation as well as on relationships between allergy and environmental factors (infections, smoking, sports). Since 1996 S. Bonini has a research appointment at the Institute of Neurobiology and Molecular Medicine, Italian National Research Council (CNR) for studies on ‘‘Modulation of allergic inflammation’’. Scientific production of Prof. Bonini includes approximately 500 scientific articles, chapters of books, proceedings of international congresses (more than 150 indexed by ISI medline). The most cited papers among those published by him and his research group include the original description of a late-phase reaction in the eye, the first report on the potential relevance of NGF in allergic inflammation and tissue remodelling, the description of genetic and environmental factors influencing allergy phe-

notypes (IgE, basophil histamine release, inflammatory cells' functions, tissue hyperreactivity). Prof. Bonini has been President (1998–2001) of the European Academy of Allergology and Clinical Immunology (Secretary General 1986–1998). He is member of several national and international scientific societies and member of the Research & Development Commission of the Italian Drug Agency.

Prof. Francesca Levi-Schaffer is Chairman of and Professor in the Department of Pharmacology and Experimental Therapeutics of the School of Pharmacy in the Faculty of Medicine at The Hebrew University of Jerusalem. She is head of the Teaching Unit of Pharmacology and Experimental Therapeutics in the Faculty of Medicine, School of Pharmacy. Prof. Levi-Schaffer holds the Isaac and Myrna Kaye Chair in Immunopharmacology, and is an honorary senior lecturer at the National Heart and Lung Institute of the Imperial College in London. She was born in Italy and completed her MSc degree in pharmacy at the University of Milano. In 1978 she completed her PhD degree in Immunology at the Weizmann Institute in Rehovot, Israel. Her post-doctoral training was at Harvard Medical School. Prof. Levi-Schaffer has published 118 articles in peer-reviewed journals, 50 reviews and editorials and 12 book chapters. She also has two provisional patents pending.

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ALLERGIC BRONCHIAL AIRWAY INFLAMMATION IN NERVE GROWTH FACTOR (NGF)-DEPRIVED RATS: EVIDENCE SUGGESTING A NEUROIMMUNOMODULATORY ROLE OF NGF

Barbara Stampachiacchiere and Luigi Aloe □ *Institute of Neurobiology and Molecular Medicine, Section of Neurobiology, National Research Council (CNR), Rome, Italy*

Alessandra Micera □ *Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Israel; and CIR Laboratory of Ophtalmology, University Campus Bio-medico, Rome, Italy*

Sergio Bonini □ *Institute of Neurobiology and Molecular Medicine, National Research Council, Rome, Italy; and Second University of Naples, Naples, Italy*

□ *In the present study, ovalbumin-sensitized/challenged rats were characterized by an nerve growth factor (NGF) increase in both serum and bronchial alveolar lavage fluid (BALF), but not in the lung. Exogenous administration of NGF or NGF-neutralizing antibodies did not modify immunoglobulin (IgE) and eosinophil parameters. In control rats, NGF administration did not induce increase of IgE or eosinophils in both BALF and lung. The present findings suggest that at least NGF does not act as a proper proinflammatory factor but most probably as a neuroimmune modulator molecule of the allergic state.*

Keywords allergy, eosinophils, inflammation, mast cells, NGF

Nerve growth factor (NGF) is a neurotrophin that, in addition to its classical nervous system domain [1], also acts on a variety of immune cells [2–9]. NGF is physiologically present in the bloodstream and tissues of mammals, humans included [10], and undergoes significant changes during neuro- and immunoinflammatory disorders [11]. The biological activity of NGF is mediated by specific binding to trkA^{NGFR} and p75^{NTR} receptors both on neural and immune cells [12].

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Address correspondence to Luigi Aloe, Institute of Neurobiology and Molecular Medicine, Section of Neurobiology, CNR, Rome, Italy. E-mail: aloe@inmm.cnr.it

We previously reported that immune cells are receptive to NGF action [3–5, 10] and that the human allergic response is characterized by a significant increase in circulating NGF [13]. Recently elevated amounts of NGF have also been found in the bronchial alveolar lavage fluid (BALF) and in the lung of an animal model of allergic inflammation [14]. This animal model is characterized by increased number of lymphocytes, eosinophils (EOS), and mast cells (MCs) into the airways, resulting in the development of a Th2 immune response, with structural changes typical of the airway hyperresponsiveness [14–17]. We first reported the increase of circulating NGF levels in patients suffering from allergic states and lung inflammation, suggesting that NGF might play a role in lung inflammation and bronchial hyperresponsiveness [2].

However, the functional significance of such an increase has not yet been clearly established, because the evidence obtained both with humans and animals indicate that NGF can exert both proinflammatory and anti-inflammatory roles [4, 6, 12–15]. The anti-inflammatory hypothesis is consistent with a number of recent reports showing that at doses as low as 10 μg/mL NGF induces interleukin (IL)-6 production and inhibits tumor necrosis factor (TNF)-α release from MCs by regulating, via prostanoid-dependent mechanisms, local inflammatory responses and degeneration after tissue injury [18]. NGF also protects against central nervous system (CNS) inflammation in a nonhuman primate model of experimental allergic encephalomyelitis (EAE) by influencing the balance between Th1 and Th2 cytokine expression in the brain [19]. Based on these studies, it was suggested that NGF can mimic asthma features by enhancing bronchial hyperactivity and allergic inflammation [15–28], or that it can decrease inflammatory responses [21–24]. However, other studies have showed that during inflammatory disorders, mast cell–derived NGF can provide a mechanism by which local harmful cytokine response might be limited [18]. Thus NGF might be an important factor in asthma-associated inflammatory responses and in other pathological conditions in which tissue inflammation or tissue repair have been observed.

Based on the different roles played by NGF on peripheral nerve cells, inflammatory cells and asthma-associated cytokines IL-1β and TNF-α, the present study was designed to further understand the role of NGF in this inflammatory disorder. We used animal models of allergic bronchial inflammation with low (NGF-deprived rats) or high levels of NGF. The rationale behind this novel experimental approach was that low circulating levels of NGF would reduce inflammatory responses, the number of immune cells of the immune system, and the concentration of cytokines that are critically important in the development of asthma-related symptoms, whereas high levels would exacerbate these responses.

MATERIALS AND METHODS

Chemicals

Ovalbumin (OVA), aluminum ($Al(OH)_3$) and cytochrome *c* (Cyt C) were purchased from Sigma (St Louis, USA). Rabbit anti-human $trkA^{NGFR}$ antibodies (2 μg/mL) were purchased from Santa Cruz (Biotechnology, CA, USA). Anti-rat $p75^{NTR}$ antibodies (Mab 192) were developed in our laboratory. Detection mouse anti-rat immunoglobulin (IgG) antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA). 2.5S NGF was purified from mouse salivary gland and purified by chromatography, according to the method of Bocchini and Angeletti [29], with slight modification. The purity of NGF was tested by electrophoresis and immunoblotting analysis, using anti-NGF antibody (Ab-NGF) against 2.5S NGF that recognize also human and rat NGF [30], and Ab-NGF antibodies from Chemicon (USA).

Animals

Pathogen-free 8-month-old female Sprague-Dawley rats (n = 42) were used for our studies [31, 32]. Rats were housed 2 per cage, under controlled conditions on a 12-hour light/dark cycle, and provided with food and water ad libitum. For housing, care, and experimental procedures, we followed the guidelines indicated by our intramural animal ethical committee, in conformity with the Institutional Guidelines in accordance with National and International law (EEC council directive 86/609, OJ L 358, 1, December 12, 1987).

OVA Sensitization and Challenges

Rats (n = 24; 6 rats/4 experimental groups) were actively sensitized by single intraperitoneal injection of 1 mg OVA preadsorbed with 1.5 mg $Al(OH)_3$ in 0.5 mL of phosphate-buffered saline (PBS) on days 0 and 7. Starting on day 14, rats were OVA challenged (2%, w/v in PBS) for 30 minutes, 3 times/day over a period of 10 consecutive days (day 24), according to a previous reported method [16]. Control rats (n = 6) received PBS. All rats were analyzed 24 hours after the last challenge (day 24). A detailed flow chart of this experimental procedure is depicted in Figure 1.

NGF Administration

Rats (n = 12; 6 rats/2 experimental groups) received a single injection of 100 μg 2.5S NGF dissolved in 100 μL of physiological solution (0.9%

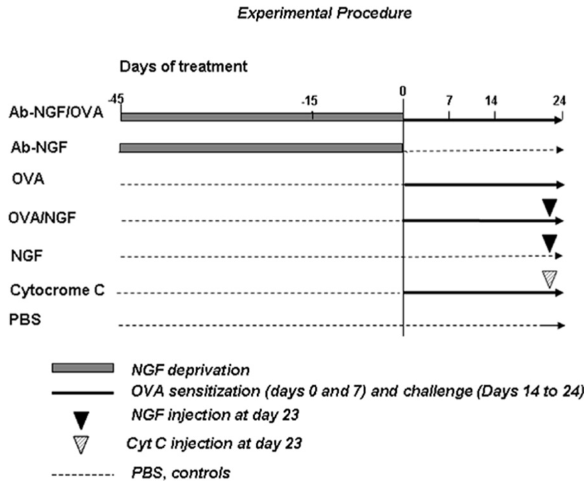


FIGURE 1 Time schedule describing the experimental procedure to induce Ab-NGF antibodies in rats, OVA immunization, and treatment with NGF. NGF = NGF treated rats (n = 6); OVA = ovalbumin-sensitized rats and (n = 6)-challenged rats (n = 6); OVA/NGF = OVA-immunized and NGF-treated rats (n = 6); Ab-NGF = NGF-deprived rats (n = 6); Ab-NGF/OVA = NGF-deprived and OVA-immunized rats (n = 6). Further details are given in Materials and Methods.

NaCl) into the tail vein. Control rats (n = 6) were injected with 100 μg of Cyt C, a molecule physiochemical similar to NGF, but lacking in its biological activity [33–36]. Both control (Cyt C) and NGF-treated rats were sacrificed 24 hours after the last treatment.

NGF Deprivation (Induction of Endogenous Ab-NGF Antibodies)

To stimulate endogenous production of Ab-NGF antibodies, anaesthetized rats were treated as previously reported [5, 37]. Briefly, rats (n = 12; 6 rats/2 experimental groups) were sensitized with 200 μg 2.5S NGF in 150 μL complete Freund's adjuvant (CFA) and 150 μL physiological solution. A booster of the same emulsion was given 4 weeks later. With this procedure, rats produce autoantibodies that cross-react with endogenous NGF [38–40]. The Ab-NGF titer was quantified using a semiquantitative Doubleday and Robinson enzyme-linked immunosorbent assay (ELISA) [41], and expressed as the highest dilution at which the absorbance was greater than 2 standard deviations above the mean of the sample assay. Briefly, 96-well plates were coated with 2 μg/mL specific NGF at 37°C for 1 hour and then different dilution of serum (ranging from 1:500 to 1:150000) were loaded to evaluate the titer of auto-antibodies. The specific bound of anti-rat NGF antibodies was detected by using biotinylated anti-rat IgG (1:8000; Zymed), horseradish peroxidase streptavidin (1:200; R&D), Tetramethyl Benzidine Solution (TMB) (Zymed), and finally read at

450 nm with an ELISA reader (Dynatech MR 5000; PBI, Germany). Each assay included 6 samples of preimmune rat plasma, which were tested at a dilution of 1:20. Ab-NGF antibody component belongs to the IgG fraction as previously reported [38, 39] (data not shown).

To assess whether Ab-NGF were able to neutralize the biological activity of NGF, the sera of NGF-deprivated rats were tested in a previously described NGF-antibody bioassay [36, 42].

Rats containing a titer of circulating Ab-NGF antibodies over 1:2000 and showing no pathological signs, such as body weight loss, skin lesion or consistent granuloma formation, at the site of NGF injection, were used for OVA sensitization/challenge.

Peripheral Blood and BALF Collection

Rats received an overdose of sodium pentorbabital (Nembutal) and blood samples were collected from the carotid cannula, clotted at room temperature, centrifuged at 1300 rpm for 10 minutes, and the resulting serum was stored until used. For BALF collection, a cannula was inserted in the trachea in situ and the lungs were lavaged with portion of 5 mL of cold 0.9% NaCl. The fluid was retrieved by gentle aspiration and this procedure was repeated 5 times. The recovery of fluid was over 80% in all rats. The recovered lavage fluids were cooled in ice and centrifuged (5 min/1300 rpm) at 4°C for 10 minutes and supernatants stored at −70°C for cytokine immunoenzymatic determination. Pellet cells were collected, mixed in distilled water (30 seconds in ice), to lyse red blood cells, resuspended in PBS, cytospunned onto glass slides (15 min/200 rpm) (Cytospin II Shandon, UK), and stained with Luxol fast blue or hematoxylin and Eosin. Stained cells were observed under Zeiss Axiophot microscope (×100 objective/immersion) and the number of EOS in counted 100 cells of different field ($n = 20$ fields/slide) of each experimental group ($n = 7$) was counted and compared.

IgE Assay

IgE antibodies were measured with a 2-side ELISA, using 2 monoclonal anti-rat IgE antibodies (Pharmingen). Plates were coated with 2 μg/mL specific antibody (10181D; Pharmingen), and then different dilutions of sera (ranging from 1:500 to 1:150000) were applied to evaluate the IgE titer. The second biotinylated antibody (10192D; Pharmingen), horseradish peroxidase–streptavidine conjugate (1:200; R&D), and TMB substrate (Zymed) were used according to the standard protocol.

OVA-specific IgE were measured by using a sandwich ELISA with OVA in the solid phase. For the assay, diluted samples were added to 10 ng/mL

OVA-precoated plates and the specific binding was detected by using biotinylated monoclonal anti-rat IgG antibodies following the standard procedure. Values are expressed as IgE dilution response to OVA stimulation.

TNF-α Assay

TNF-α activity in the serum, BALF, and lung was determined by a cytotoxicity assay using the TNF-α–sensitive WEHI 164 clone 13 cell line [43]. In brief, the cells (2.5×10^5 cells/mL) were cultured in monolayer in 96-well NUNC plates, under standard conditions. Seventy percent to 80% of confluent actinomycin D cells were incubated with TNF-α standards (ranging from 0.12 pg/mL to 2000 pg/mL), 100 μL of serum (1:10), BALF (1:10), or lung homogenates (1:20). After 20 hours, incubation, a solution of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; G4000; Promega, Medison, USA) was added for 4 hours at 37°C, developed with 10% sodium dodecyl sulfate (SDS) solution in 0.01 N HCl, and read at 570 nm in an ELISA reader (Dynatech MR 5000). The amount of biologically active TNF-α in the tested samples was calculated on the basis of the standard curve, and expressed as pg/mL (blood and BALF) or pg/g tissue (lung).

NGF and BDNF ELISA

The level of NGF was measured by a highly sensitive 2-site immunoenzymatic assay (ELISA), following the manufacturer's instructions reported in the kit (G7630; Promega). Blood and BALF samples were 1:4 diluted in lysis buffer (10 mM phosphate buffer [PB], 150 mM NaCl, 10 mM EDTA, 10 mM benzetonium chloride, 2% gelatin, 10 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% Triton, and 0.1% bovine serum albumine [BSA]). Lung tissues were extracted by sonication (40 pulse/s) at the concentration of 250 mg/mL in lysis buffer. Protein amounts, evaluated after sonication, were assayed by the modified Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). NGF sensitivity was 3 pg/g (with no cross-reactivity with the other neurotrophins), and the recovery ranged from 80% to 90%. Data are presented as pg/g (wet weight) for lung or pg/mL for serum and BALF and all assays were performed in triplicate.

The levels of BDNF were detected by using a commercially available specific ELISA kit (G7611; Promega) as previously described [37]; the sensitivity of the assay was 15 pg/g Brain Dervied Neurotrophic Factor (BDNF) and cross-reactivity with other related neurotrophic factors (NGF, NT-3, and NT-4) was less than 3%. Data are represented as pg/g wet weight for

lung, pg/mL for serum and BALF, and all assays were performed in duplicate.

Histological Evaluation

Lungs were fixed with 4% paraformaldehyde, or Bouin fixative, and 20-μm sections were cut with a cryostat (Microm Biopticam, Milan, Italy). Sections were stained with Luxol fast blue (Sigma), counterstained with hematoxylin (Sigma), and observed (under a blind fashion) under Zeiss Axiophot microscope (×100 objective/immersion). The number of EOS present in 100 total cells per field (n = 20 field/slide/rat) of each sections (n = 6 section/slide/rat) and experimental group (n = 4 rats/experimental group) was counted, compared, and expressed as percentage of EOS. Lung sections were also stained with toluidine blue or May-Grundwald for identifying MCs distribution.

Statistical Analysis

Data are expressed as median and range, or as mean ± SEM of 3 independent experiments in which the different groups were tested in triplicates or quadruplicates. Nonparametric analysis (analysis of variance [ANOVA], followed by Tukey-Kramer posthoc test) was used to compare the effects. In both cases, a probability of $\leq .05$ was considered statistically significant. The statistical package used was STATVIEW II for Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

As reported by others [31, 32, 44], OVA-sensitized and -challenged rats developed the histopathological and biochemical features of asthma (see below). NGF-depriveted rats produced circulating Ab-NGF that were able to neutralize the biological activity of NGF, at dilution equal to a titre of 2000 or greater. All rats, developing asthma and/or producing Ab-NGF, displayed normal behavior, maintained normal body weight, and showed no evident macroscopic pathological signs.

OVA Immunization and Clinical Symptoms

After OVA treatment, the daily observation showed no differences in allergic manifestation between OVA rats and Ab-NGF/OVA rats. Both groups displayed the typical allergic behavioral response after OVA immunization, and all survived for the entire experimental period. OVA rats

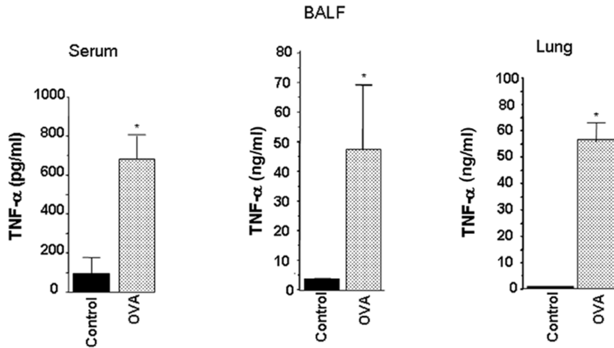


FIGURE 2 Levels of TNF-α in the serum, BALF, and lung of rats treated with OVA as compared to control values. Statistically significant differences were found in the serum ($^*P = .01$), BALF ($^*P = .01$), and lung tissues ($^*P < .01$) of OVA-treated rats as compared to controls.

developed OVA-specific IgE, indicating the effectiveness of the treatment. Figure 1 illustrates the protocol used for this experimental investigation.

Effect of OVA Immunization on TNF-α Levels and MCs Recruitment

Because TNF-α is known to increase in allergic diseases, mainly indicated as a proinflammatory marker of inflammation [24, 25], the level of TNF-α in the serum, BALF, and lung of OVA rats was investigated and compared to the levels in Ab-NGF or NGF treatments. As shown in Figure 2, OVA rats displayed increased TNF-α levels in the serum (629% increase over basaline; $P < .01$), BALF (1236% increase over basaline; $P < .01$), and lung tissue (22168% increase over basaline; $P < .01$). No significant difference was observed in TNF-α levels among NGF (serum: 227%; BALF: 1294%; lung: 1308%; $P > .05$), OVA/NGF (serum: 727%; BALF: 1179%; lung: 20100%; $P > .05$) and Ab-NGF/OVA (serum: 116%; BALF: 400%; lung: 317%; $P > .05$) rats, compared to those of controls. Histological and cytological analysis of BALF and lung revealed that OVA-induced inflammation caused an increase in the number of inflammatory cells (EOS and MCs) in the BALF and in the lung (not shown). As shown in Figure 3, an increase in MC number was found in the lung of OVA, OVA/NGF, and Ab-NGF/OVA rats as compared to NGF and Ab-NGF rats, respectively.

Effect of OVA Immunization on Circulating and Local NGF Levels

To investigate the role of NGF in this experimentally induced inflammatory event, we measured the amount of NGF in the blood and respiratory fluid/tissues. As shown in Figure 4, the inflammatory response in

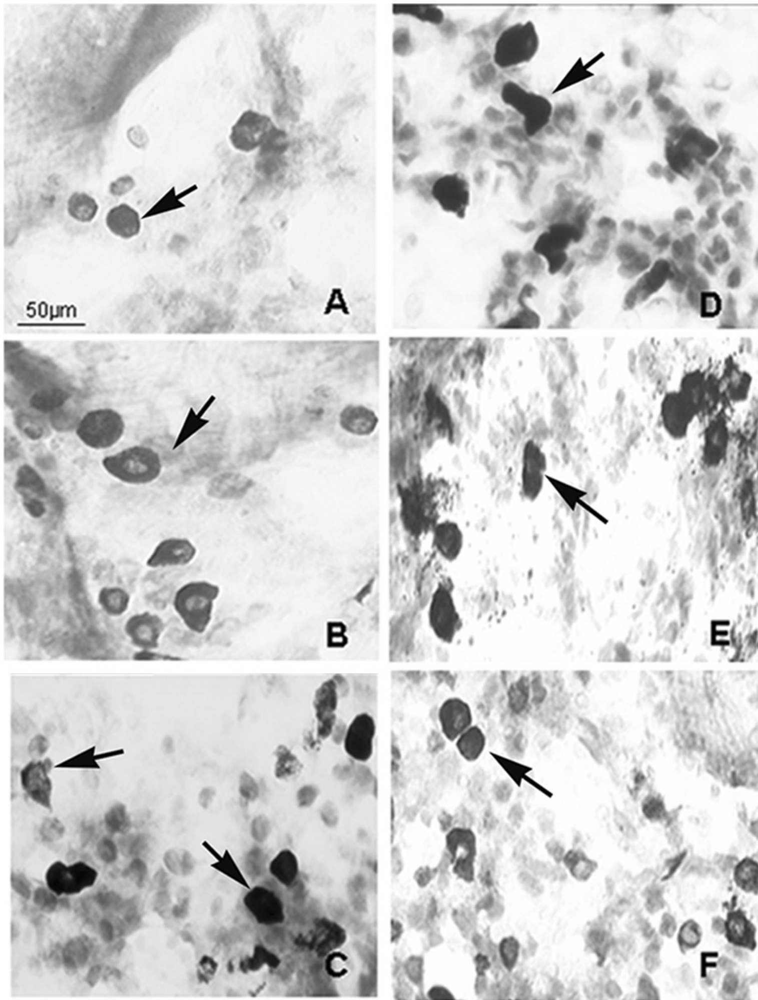


FIGURE 3 Mast cells distribution in the lung tissues of untreated rats (*A*) and rats treated with NGF (*B*), Ab-NGF (*C*), OVA (*D*), OVA/NGF (*E*), Ab-NGF/OVA (*F*). An increase of mast cell number is observed in OVA (*D*), OVA/NGF (*E*), and Ab-NGF/OVA (*F*) groups, as compared to control (*A*), NGF (*B*), and Ab-NGF (*D*) groups. Toluidine blue–stained sections. ×250.

OVA rats caused a significant increase in NGF in the serum (50% increase over baseline; $P < .05$) and BALF (169% increase over baseline; $P < .01$), but not in the lung (4.8% increase over baseline; $P > .05$).

Effect of Circulating NGF or Ab-NGF Antibodies on BALF EOS in OVA Rats

All Ab-NGF/OVA rats developed allergic symptoms similar to OVA rats. In order to obtain additional insight in the role and the significance of the

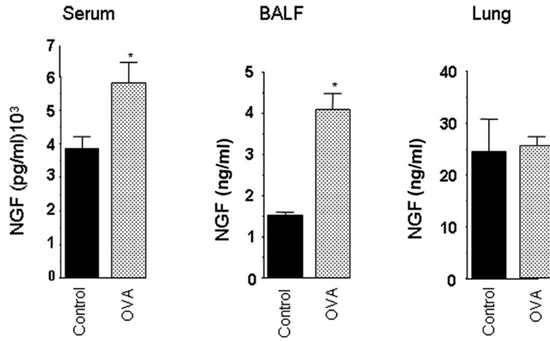
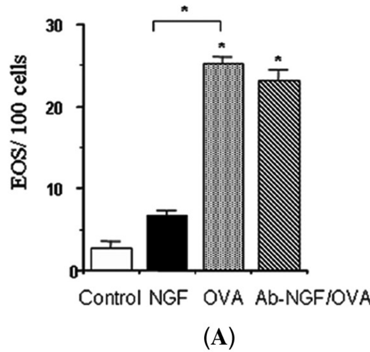
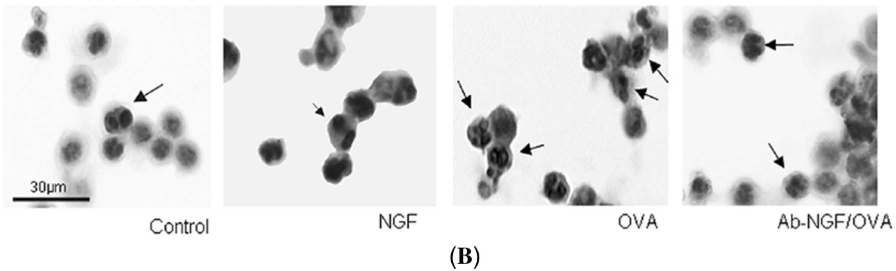


FIGURE 4 NGF concentration in the serum, BALF, and lung of rats treated with OVA, showing significant increase of NGF in the serum ($^*P < .05$), BALF, ($^*P < .01$) but not the lung ($P > .05$), as compared to controls.

enhanced circulating NGF after allergic bronchial asthma, we compared the distribution of EOS, in the BALF and lung of OVA and Ab-NGF/OVA rats. As reported in Figure 5*A*, EOS% in the BALF increased slightly after NGF treatment and to a greater extent in the BALF of OVA rats (837%



(A)



(B)

FIGURE 5 Quantitative (*A*) and qualitative (*B*) presence of eosinophils (EOS) in the BALF of adult rats treated as indicated. Cells were stained with hematoxylin and eosin and the number of EOS counted using a Zeiss microscope with a ×100 objective. Values are expressed as number of EOS per 100 total cells; $^*P < .01$ (*A*). EOS are indicated by arrows. ×450.

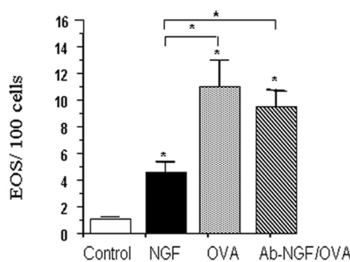
increase over control; $P < .01$). Circulating Ab-NGF antibodies did not reduce the number of EOS in the BALF, as compared to OVA rats.

Effect of Circulating NGF or Ab-NGF Antibodies on Lung EOS in OVA Rats

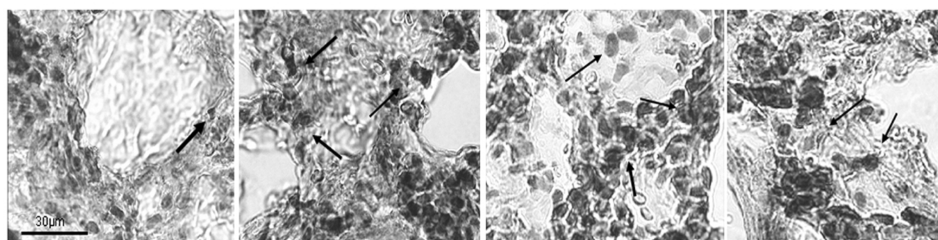
As shown in Figure 6 and according to BALF results, the number of EOS (for optic field) increased in the lung of NGF (283% increase over control; $P < .05$) and OVA rats (816% increase over control; $P < .01$). Circulating Ab-NGF antibodies did not reduce significantly the presence of EOS in the lung (15.9% decrease over OVA treated rats; $P > .05$). These observations suggest that the increase of EOS in both BALF and lung is not directly associated with the elevated presence of NGF.

Effects of NGF on IgE Levels in Control and OVA Rats

Finally, to firstly investigate the action of NGF in allergic bronchial response, we measured the amount of IgE present in the serum and BALFOVA rats with elevated circulating levels of NGF or Ab-NGF antibodies.



(**A**)



(**B**)

FIGURE 6 Quantitative (*A*) and qualitative (*B*) presence of eosinophils (EOS) in the lung of adult rats treated as indicated, stained with Luxol fast blue, and examined with Zeiss microscope equipped with a ×100 objective. EOS were counted in each experimental groups: the number of EOS were found significantly increased (indicated by *arrows*), as compared to controls, in all 3 groups (*$P < .01$), ×400.

TABLE 1 IgE Levels in the Serum and BALF of Systematically OVA-Immunized Rats

Treatment	Serum	BALF
Control (PBS)	1:200–1:500	1:200–1:500
NGF	1:500–1:2000	1:200–1:500
OVA	1:32000–1:64000	1:4000–1:8000
OVA/NGF	1:32000–1:64000	1:4000–1:8000
Ab-NGF/OVA	1:4000–1:8000	1:500–1:2000

Note. OVA-specific IgE levels of the test sample were determined as the highest dilution at which the absorbance was greater than 2 standard deviations above the mean of the samples assay.

As reported in Table 1, the administration of NGF caused a mild increase in IgE in the serum (1:2000 versus 1:500), but not in BALF (1:500 versus 1:500), of control rats, whereas OVA and OVA/NGF displayed a consistent increase in IgE in the serum (1:64000 versus 1:500; 1:64000 versus 1:500) and a less marked increase in the BALF (1:8000 versus 1:500; 1:8000 versus 1:500). Interestingly, the presence of circulating Ab-NGF antibodies significantly decreased IgE levels both in the serum and BALF.

DISCUSSION

Published evidence on animal models and humans showed that NGF increases after allergic inflammatory responses [4, 13, 14, 17]. The functional significance of such an increase is not clearly known. In the present study, we induced an allergic bronchial inflammation in NGF-deprived rats (Ab-NGF/OVA rats) to investigate whether the inhibition of endogenously released NGF might affect inflammatory/allergic markers. We found that OVA rats developed asthma-like responses, characterized by increased levels of IgE and TNF-α in the serum and BALF, and by significantly high levels of NGF in the BALF, but not in the serum or in the lung. OVA rats were also characterized by an increase in EOS in the BALF and lung tissues. However, when bronchial inflammation was induced in NGF-deprived rats, a reduction of NGF in the serum and BALF was observed, but not in the levels of IgE, TNF-α and EOS. This study showed that specific NGF deprivation did not affect the allergic inflammatory response observed in OVA rats, which are characterized by high levels of IgE, TNF-α, and NGF as well as increased local MCs and EOS. This would imply that NGF does not exert a direct role in allergic inflammatory events. This observation is consistent with the fact that an exogenous injection of NGF in control and OVA rats did not enhance the IgE levels in the serum and BALF. No changes in the BDNF levels in the lungs and BALF of OVA rats were found. It remains unclear why lung and BALF respond differently to NGF levels in OVA rats. One reasonable hypothesis is that different tissues do not respond in similar ways to circulating NGF or Ab-NGF, and NGF has different effects on a

number of cells, such as neurons, smooth muscle and immune cells [17]. In allergic diseases the cellular origin of increased levels NGF is not clear, though the available evidence suggests that MCs, migrated blood cells (EOS), local fibroblasts, or epithelial cells might be some of the main sources of NGF [4, 6, 11, 17, 45, 46]. The elevated amount of NGF observed in the fluids and tissues might be the result of a reduced number and/or capacity of NGF receptive cells located in the respiratory system [47].

A major finding of this study is the observation that the drastic reduction or near absence of circulating NGF in NGF-deprived rats did not modify the histopathological and biochemical responses in OVA rats. These observations seem therefore to support the hypothesis that NGF might be important to maintain structural and functional integrity of neuro-immune tissue remodeling during airway inflammation [14].

Recent studies have shown that in the induction of murine allergic airway inflammation and asthma NGF promotes or exacerbates inflammatory response [14, 15, 20–23], whereas the present findings suggest that NGF has an anti-inflammatory effect, supporting the hypothesis that NGF might be involved in promoting chronic inflammatory related healing [22, 23, 25, 48, 49]. Though the mechanism through which NGF exerts this effect is unclear, the evidence that sensory nerves regulate many aspects of airway function, including smooth muscle tone, bloodflow, airway secretion, microvascular leakage, and the release of mediators from inflammatory lung cells, suggests that the NGF released during allergic responses might be involved in neuroregulatory processes [4, 11]. This role might not be restricted to allergy, but can occur in other inflammatory conditions [17]. Indeed, NGF has been shown to have anti-inflammatory and healing properties in some inflammatory disorders, such as experimental allergic encephalomyelitis [19, 37, 50], carrageenan-induced inflammation [25, 24], and corneal autoimmune inflammation [45, 48].

NGF is essential not only for the survival of sensory and sympathetic nerve, cells, but also for cells of the immune system [1–11]. At doses as low as 10 μg/mL, NGF induces IL-6 production and inhibits TNF-α release from MCs by regulating, via prostanoid-dependent mechanisms, local inflammatory responses and degeneration after tissue injury [18]. Evidence suggest both the anti-inflammatory action as well as NGF modulation of repair by increasing the rate at which fibroblasts migrate in response to chemoattractants [17, 49]. It is therefore possible that, even in the presence of other cytokines, NGF can be considered a positive-negative mediator and that the balance of these two effects could have a proinflammatory or anti-inflammatory action. It should also be taken into consideration that NGF, through its regulatory action on peripheral sympathetic and sensory nerves, can decrease the associated damaging effects of inflammation on peripheral nerve endings and on specific subpopulation of immune cells [1–11]. This

NGF action can be influenced by the amount of NGF released and on the specific stage of inflammation. The hypothesis of an anti-inflammatory role of NGF is also consistent with recent reports showing that NGF exerts anti-inflammatory action in rodents, nonhuman primates, and in humans [19–23, 26, 27, 35, 45, 50]. NGF also protects against CNS inflammation in a nonhuman primate model of EAE by influencing the balance between Th1 and Th2 cytokine expression in the brain [19], and has anti-inflammatory and healing properties in cases of immune corneal ulcers with stromal melting [45, 48].

Nonetheless, in order to establish whether NGF promotes, exacerbates, or decreases bronchial airway inflammation, further biochemical, pharmacological, and molecular experimental strategies remain to be done with this animal model. One possible experimental strategy to address this question would be to treat the animal model of airway inflammation with specific agonist or antagonist involved in inflammatory responses and monitor the biochemical and molecular changes of endogenous NGF determinants.

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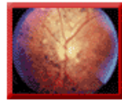

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Human Idiopathic Epiretinal Membranes Express NGF and NGF Receptors

Author(s): MINCHIOTTI, SIMONA MD*; STAMPACHIACCHIERE, BARBARA MD*; MICERA, ALESSANDRA PhD*†; LAMBIASE, ALESSANDRO MD, PhD†; RIPANDELLI, GUIDO MD*†; BILLI, BERNARDO MD*†; BONINI, STEFANO MD†

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Institution(s): From *IRCCS-G.B. Bietti Eye Foundation; and †CIR, Laboratory of Ophthalmology, University Campus Bio-Medico, Rome, Italy.

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Reprint requests: Stefano Bonini, MD, Department of Ophthalmology, University "Campus Bio-Medico", Via Alvaro del Portillo 21, 00128 Rome, Italy; e-mail: s.bonini@unicampus.it

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Abstract

Purpose: Glial cells and fibroblasts (FBs) play a key role in epiretinal membrane (ERM) development and progression. Myofibroblasts (myoFBs), arising from these cells, can lead to the hypertrophic scars and tissue contraction observed in ERMs. Nerve growth factor (NGF) and transforming growth factor-[beta]1 (TGF-[beta]1) play a crucial role in FB activities. Therefore, the authors evaluated myoFBs in ERMs and NGF, $trkA^{NGFR}$ and $p75^{NTR}$ expression, as well as TGF-[beta]1/TGF-[beta]RII levels in both ERMs and vitreous.

Methods: Eight idiopathic ERMs and vitreous were obtained from patients at the time of vitrectomy for macular pucker. Ten control vitreous were from donors. Biochemical and molecular analyses were performed to identify [alpha]-smooth muscle actin ([alpha]-SMA, a defined myoFB marker), NGF, $trkA^{NGFR}/p75^{NTR}$, and TGF-[beta]1/TGF-[beta]RII.

Results: Every idiopathic ERM displayed [alpha]-SMA positive myoFBs, expressing NGF, $trkA^{NGFR}$, and $p75^{NTR}$. ERM vitreous showed a significant decrease in NGF protein coupled with a TGF-[beta]1 increase. In addition, vitreous cells showed an increase in $trkA^{NGFR}/p75^{NTR}$ mRNA associated with a decrease in TGF-[beta]RII mRNA.

Conclusions: Idiopathic ERMs were characterized by myoFBs. The expression of NGF, $trkA^{NGFR}$, and $p75^{NTR}$ in local myoFBs associated with changes in ERM vitreous NGF suggests an involvement of NGF, as previously reported for TGF-[beta]1, in the evolution and myoFB-mediated contractile activity of ERMs.

Epiretinal membranes (ERMs) derive from fibrocellular proliferation on the surface of the internal limiting membrane and are characterized by cell-mediated contraction that occurs idiopathically or as a secondary process of diseases such as proliferative vitreoretinopathy or diabetic retinopathy.[1-4] Patients with idiopathic ERMs may have significant macular dysfunction, due to membrane contraction, and may complain of marked metamorphopsia, severe loss of visual acuity, and occasional central binocular diplopia.[2]

Retinal pigment epithelium (RPE) cells, macrophages, fibroblasts (FBs), glial cells, and vascular endothelial cells are visible in ERMs.[5] Glial cells are typical of idiopathic ERMs, together with RPE, macrophages, FBs, and myofibroblasts (myoFBs). MyoFBs may originate from any or all of the other cell types.[5-8] All cells involved in the development of ERMs migrate through defects in the internal limiting membrane and proliferate/differentiate on the retinal surface [9] as a result of physical and/or biochemical changes in the surrounding milieu.[10-12]

Growth factors, cytokines, and overt extracellular matrix deposition are involved in the tissue remodeling process that occurs in several chronic diseases and in the genesis of ERMs.[13-16] Although transforming growth factor-[beta]1 (TGF-[beta]1) is the most important pro-fibrogenic molecule,[14,17-19] increasing evidence suggests that nerve growth factor (NGF) also has pro-fibrogenic activity.[14,20] NGF is a pleiotropic factor with many physiologic and pathologic roles in the nervous, immune, endocrine, and visual systems. NGF acts via specific binding to $trkA^{NGFR}$ and $p75^{NTR}$.[13,14,19-24] Biologic effects of NGF include FB migration, differentiation into myoFBs ([alpha]-smooth muscle actin [[alpha]-SMA] expressing cells), and extracellular matrix contraction as observed in cultured skin, lung, and conjunctival FBs.[14,19] With distinct pro-fibrogenic activity, NGF and TGF-[beta]1 appear to cooperate during the complex phases of tissue repair/remodeling.[19]

Although NGF and TGF-[beta]1 expression has been shown in secondary ERMs, few studies reported their expression in idiopathic ERMs.[13,25,26] In particular, the role of NGF has not been fully investigated in idiopathic ERMs, which appear more complex in terms of development as compared to secondary ERMs, and lack medical treatment.[27]

In the present study, we sought to investigate the presence of myoFBs and the expression of NGF, $trkA^{NGFR}$, and $p75^{NTR}$ in idiopathic ERMs and vitreous, compared to the expression and activity of TGF-[beta]1.

Materials and Methods

This study followed the guidelines of the Declaration of Helsinki for research involving human subjects. Informed consent was obtained from each patient or his or her parents.

All sterile plasticware and analytical grade reagents were obtained from NUNC (Roskilde, Denmark), SERVA (Heidelberg, Germany), and ICN (Costa Mesa, CA) when not specified differently in the text.

Patients and Tissue Handling

Eight idiopathic macular puckers (4 M, 4 F; range 52-70 years of age) and respective vitreous samples were obtained at the time of standard pars plana vitrectomy

surgery with horizontal scissors or membrane pick. The sampling was performed in our department in the period between July 2005 and June 2006. Presurgery examination included visual acuity, Amsler's test, biomicroscopy, indirect ophthalmoscopy including scleral depression, and optical coherence tomography (Stratus OCT; Carl Zeiss, Germany). Exclusion criteria were as follows: 1) presence or history of other ocular diseases; 2) history of previous retinal treatments; 3) history of previous eye surgery; and 4) diagnosis of retinal tears or holes. A flow chart summarizing the clinical characteristics of patients enrolled in the study is shown in Table 1.

Table 1. Clinical Data of the Patients With Diagnosis of Idiopathic ERM

Patient	Sex/ Age, yr	Symptoms	Duration, mo	Best-Corrected VA	Anterior Segment Examination	Fundus Examination	Amsler Test	OCT:Central Macular Thickness, μm (nv = 147 ± 17 μm)
1	F/65	L: metamorphopsia, blurring of central vision	12	R: 20/20; J2 L: 20/63; J8	R + L: normal	L: PVD, macular pucker, and macular edema	P	L: 487 ± 17
2	M/57	R: metamorphopsia; reduction of central vision	8	R: 20/100; J8 L: 20/20; J2	R + L: normal	R: PVD, macular pucker, and macular edema	P	R: 517 ± 11
3	M/55	R: blurring of vision L: metamorphopsia, blurring of central vision	3 (R) 6 (L)	R: 20/32; J5 L: 20/100; J10	R + L: normal	R + L: PVD, macular pucker, and macular edema (L > R)	P	R: 363 ± 7 L: 545 ± 5
4	F/52	R: blurring of central vision	18	R: 20/200; J17 L: 20/32; J2	R + L: lens wedge-shaped opacities	R: PVD, macular pucker, and macular edema	P	R: 514 ± 11
5	M/63	R + L: blurring of central vision, metamorphopsia	6	R: 20/100; J7 L: 20/32; J5	R + L: Normal	R + L: PVD, macular pucker, and macular edema (R > L)	P	R: 501 ± 13 L: 434 ± 5
6	M/70	R + L: blurring of central vision, metamorphopsia	10	R: 20/100; J10 L: 20/25; J7	R + L: lens radial spoke-like opacities	R + L: PVD, macular pucker, and macular edema (R > L)	P	R: 512 ± 13 L: 345 ± 11
7	F/52	R: blurring of central vision, metamorphopsia	7	R: 20/63; J8 L: 20/20; J2	R + L: lens wedge-shaped opacities	R: PVD, macular pucker, and macular edema	P	R: 493 ± 17
8	F/70	R + L: blurring of central vision, metamorphopsia	6 (R) 10 (L)	R: 20/32; J5 L: 20/200; J10	R + L: cortical cataract	R + L: PVD, macular pucker, and macular edema (L > R)	P	R: 401 ± 7 L: 514 ± 11

VA = visual acuity; P = positive; PVD = posterior vitreous detachment; nv = normal value.

Table 1. Clinical Data of the Patients With Diagnosis of Idiopathic ERM

As a control, 10 vitreous samples were collected from cadavers (6 M; 4 F; range 25–80 years of age) with no history of retinal disease or previous eye surgery at the time of multiorgan explant.

Each excised ERM was cut into two pieces: one piece was quickly processed for molecular analysis while the other was postfixed in 10% formalin and stored at 4°C until processed for light or confocal microscopy. Vitreous samples were centrifuged at 13,000 rpm/7 minutes: the supernatant was assayed for protein analysis while the pellet was used for PCR analysis.

Light and Confocal Analysis

ERM specimens were imbedded in paraffin, sectioned (5 μm; HM325 Microtome, Microm Bioptica, Milan, Italy), and processed for immunofluorescence according to the standard procedure.

Slides were incubated overnight at 4°C with single or combination of the following primary antibodies: rabbit antihuman trkA^{NGFR} (2 μg/mL; Santa Cruz Biotech, CA), goat antihuman p75^{NTR} (1 μg/mL; Santa Cruz Biotech, CA), mouse antihuman NGF (2 μg/mL; Santa Cruz Biotech), mouse antihuman [alpha]-SMA (1/50, Novocastra, Milan, Italy), and mouse antihuman TGF-[beta]1 (2 μg/mL; Santa Cruz Biotech). Specific binding of primary antibodies was detected using Cy2 or Cy3 conjugated [F(ab)$_2$] secondary antimouse (for [alpha]-SMA), antigoat (for p75^{NTR}), or antirabbit (for trkA^{NGFR}) antibodies and incubated at room temperature for 45 minutes (all from Jackson Research Laboratories, West Grove, PA). Slides were mounted in antifade medium (Vectashield, Vector Laboratories, Inc., Burlingame, CA) and examined at ×20–60/objective using a E2000U inverted confocal microscope equipped with three lasers and a DAPI lamp (Nikon, Tokyo, Japan). Images were acquired with C1 software (Nikon) and prepared with the Adobe Photoshop 7.0 program (Adobe Systems Inc., San Jose, CA). As controls, irrelevant isotype-matched IgG antibody incubations were carried out in parallel (Vector).

Parallel sections were processed for routine histology with hematoxylin and eosin (H&E, Bioptica, Milan, Italy) to evaluate the presence of structural cells and infiltrates in the ERMs. Slides were mounted (Aquamount, Vector) and examined with a ×40/objective using an Eclipse E400 Nikon microscope equipped with a light acquisition system (Lucia G Software; Nikon). Brightness/contrast levels and figure preparation were carried out with the Adobe Photoshop 7.0 program.

NGF and TGF-[beta]1 ELISA

To evaluate NGF in both vitreous and ERMs, a two site specific ELISA, with a sensitivity of 0.5 pg/mL, was performed following the Weskamp and Otten procedure, with minor modifications.28 In brief, ELISA plates (Maxisorp NUNC 96well-plates) were coated with mouse anti-NGF antibodies as a capture layer (0.4 mg/mL; MAB5260, R&D, Minneapolis, MN). After blocking treatments, the standards ranged from 0.15 pg/mL to 1 ng/mL NGF (Alomone, Jerusalem, Israel), and the diluted samples were incubated overnight at 4°C. The specific labeled was labeled using purified biotinylated polyclonal anti-NGF antibodies (0.15 μg/mL; 500-P85t; Peprotech, Milan, Italy) and a streptavidin solution (1/300; R&D). After the addition of 3,3',5,5-tetramethylbenzidine (TMB, Zymed, San Francisco, CA), the optical density at [lambda]450 (corrected for [lambda]550) was measured by an ELISA plate reader (Sunrise; Tecan Systems, Inc., San Jose, CA).

TGF-[beta]1 levels were measured in the same samples by using a commercially available ELISA kit, according to the manufacturer' procedures (Biosource International, Camarillo, CA). In both assays, protein normalization was achieved using the DC protein assay kit (Bio-Rad Laboratories, Inc, Hercules, CA).

Total RNA, cDNA Preparation, and Relative Real-Time PCR

Total RNA (tRNA) was extracted from ERMs and vitreous samples by an RNA extraction kit (MIRVANA™ Paris; Ambion, Austin, TX). Samples were pretreated with 15 μL proteinase K (20 mg/mL; Finnzyme, Milan, Italy) for 1 hour at 56°C under stable shaking, to increase cell disruption and total RNA recovery.

Total RNA samples were size-fractionated in a 1% agarose gel containing ethidium bromide (0.5 μg/mL) and photographed by a Kodak imager station (Kodak 550, Eastman Kodak Company, Sci. Imaging Systems, Rochester, NY) to verify the absence of RNA degradation. Total RNA was quantified and only samples with a rate of [lambda]$_{260}$/[lambda]$_{280}$ >1.8 were included in the study. Three μg total RNA were reverse transcribed in 20 μL final volume, using 50 pM oligo dT$_{21}$-primer (Promega, Milan, Italy), 1 mmol/L dNTP mix (Promega), and 200 U reverse transcriptase (M-MLV; Finnzyme). The reaction was carried out in a programmable PTC100 thermocycler (MJ Research, Watertown, MA).

cDNAs were run for amplification with primers specific for NGF, trkA^{NGFR}, p75^{NTR}, TGF-[beta]1, and [alpha]-SMA designed (possibly one intron-spanning) by the primer3 software available online at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (Table 2).29 GenBank software was used to select the mRNA complete sequence of each gene investigated (http://www.ncbi.nlm.nih.gov/Genbank ; provided to the public domain by the National Center for Biotechnology Information, Bethesda, MD). PCR experiments were carried out in a final volume of 20 μL containing 3μL cDNA for target genes (NGF, trkA^{NGFR}, p75^{NTR}, TGF-[beta]1, [alpha]-SMA) or 1 μL for the reference gene (GAPDH) and 17 μL master mix containing 10 μL of 10xSYBR Green PCR Mix (Applied Biosystems, Foster City, CA), 0.5 μL of each primer (10 pM, prepared by MWG, Biotech, Ebersberg, Germany), and 6 μL (for target) or 8 μL (for referring) DEPC-treated water. The PCR amplification profile, carried out in an Opticon2 MJ thermocycler (MJ Research), included 1 cycle of 95°C/15 minutes ("hot start" enzyme activation), followed by 47–55 cycles of denaturation at 94°C/15 seconds, annealing at the specific temperature (see Table 2) and extension at 72°C/30 seconds, completed by a further extension at 75°C/5 minutes. Melting

curves for each specific primer were analyzed by elevating the temperature from 56°C to 94°C while monitoring fluorescence. Samples were amplified with GAPDH primers for determination of the relative initial quantity of cDNA in each sample, and all PCR products were normalized to that amount. Negative controls (without template or with total RNA) were carried out for each run.

Table 2. Characteristics of the Primers Used in PCR Studies

Primer	5′ to 3′ direction	Amplicon (bps)	Annealing Set	Genbank Access No.
NGF	for: 5′- CTG GCC ACA CTG AGG TGC AT-3′ rev: 5′- TCC TGC AGG GAC ATT GCT CTC-3′	120	53°C, 30 sec	V01511
trkA^{NGFR}	for: 5′-CAT CGT GAA GAG TGG TCT CCG-3′ rev:5′-GAG AGA GAC TCC AGA GCG TTG AA-3′	102	57°C, 25 sec	M23102
p75^{NTR}	for: 5′- GAG GCA CCA CCG ACA ACC TC-3′ rev: 5′- TGC TTG CAG CTG TTC CAC CT-3′	147	55°C, 25 sec	AF187064
TGF-β1	for: 5′-TCC TGG CGA TAC CTC AGC AA-3′ rev: 5′-GCC CTC AAT TTC CCC TCC AC-3′	110	53°C, 30 sec	BC017288
TGF-βRII	for: 5′- GGA TTG CTC ACC TCC ACA GT-3′ rev: 5′-TCA CAC AGG CAG CAG GTT AG-3′	121	60°C, 25 sec	BC040499
α-SMA	for: 5′-GAA GGA GAT CAC GGC CCT A-3′ rev: 5′-ACA TCT GCT GGA AGG TGG AC-3′	125	57°C, 25 sec	BC017554
GAPDH	for:5′-GAA GGG GTC ATT GAT GGC AAC-3′ rev:5′-GGG AAG GTG AAG GTC GGA GTC-3′	100	53°C, 30 sec	BC013310

Table 2. Characteristics of the Primers Used in PCR Studies

Statistical Analysis

Samples were amplified in duplicate and from these replicates, means and relative amplicon values were calculated. Quantitative data were obtained from threshold cycle values (Ct), i.e., where a significant increase in fluorescence was first detected. Each sample was normalized for internal GAPDH content. According to the REST© software (available at http://nar.oupjournals.org/cgi/content/full/30/9/e36 30), C(t) were expressed as the Nfold difference (increase or decrease) in target gene expression, normalized to reference gene expression and compared to controls. The specificity of each PCR product (amplicon) was confirmed by the single melting curves obtained during amplification.

Data were analyzed for significant differences ($P < 0.05$) using the statistical package StatView II for PC (Abacus Concepts. Inc., Barkley, CA). Analysis of variance was performed using appropriate Tukey-Kramer post hoc. 31

Results

Idiopathic ERMS Contained [alpha]-SMA Positive MyoFBs

H&E staining showed the presence of different cell types in ERMs, including several macrophages and cells morphologically resembling FBs (data not shown). Specific [alpha]-SMA confocal analysis (Figure 1A) confirmed the presence of myoFBs. 13 Semiquantitative PCR confirmed the presence of [alpha]-SMA mRNA in each ERM examined (Figure 1C).

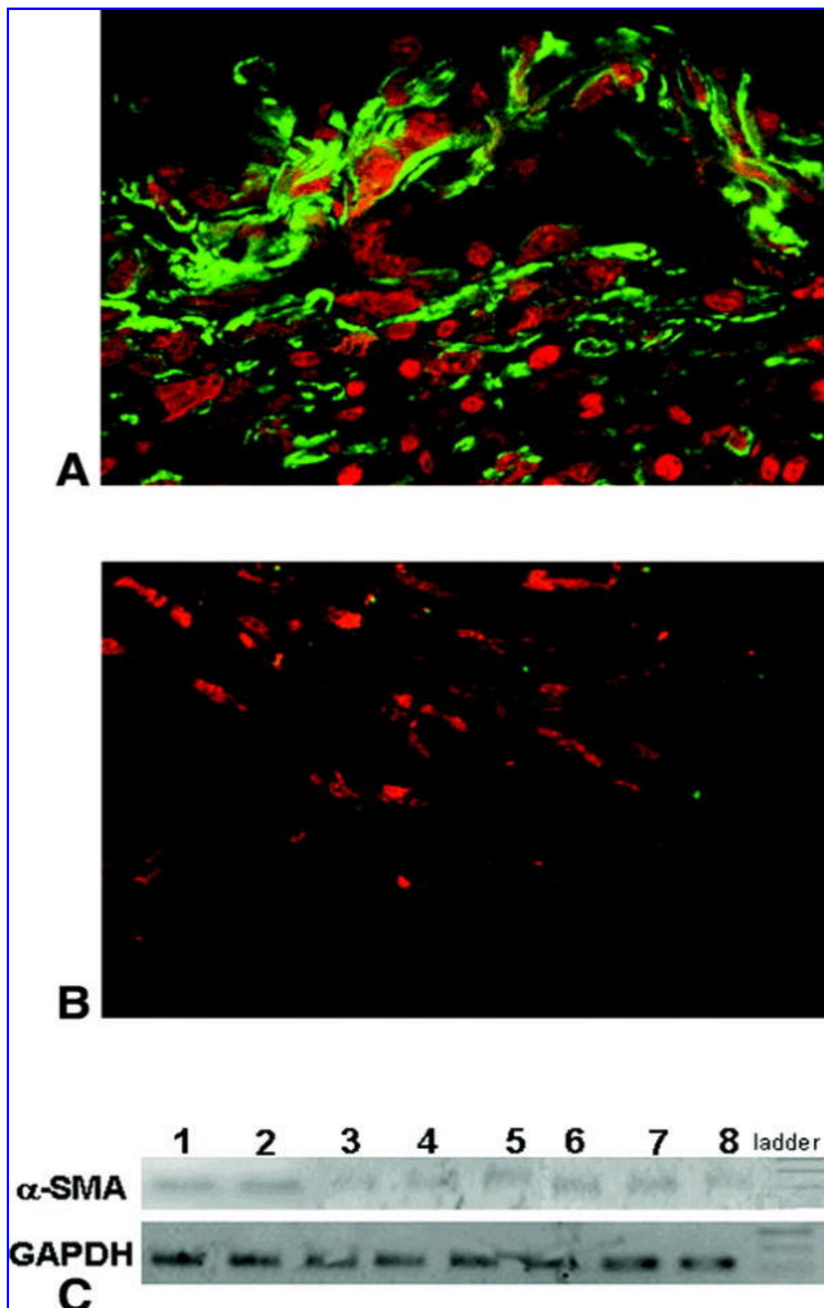

Fig. 1. Idiopathic ERMs express myoFBs. **A,** Confocal microscope analysis of an ERM section showing the expression of [alpha]-SMA (Cy2/green). **B,** Isotype control ERM section showing the specificity of the staining. Nuclei were counterstained with propidium iodide (red). Magnification ×200. **C,** Semiquantitative RT-PCR showing [alpha]-SMA mRNA PCR products (125 bps) amplified from each ERM and separated on agarose-ethidium gel. Data were normalized to GAPDH expression (100 bps).

ERMs Expressed NGF, TGF-[beta]1, $TrkA^{NGFR}$, and $P75^{NTR}$

As shown in Figure 2A, both NGF mRNA and TGF-[beta]1 mRNA were found in all ERMs evaluated. The molecular data were confirmed by confocal analysis showing the local expression of both proteins (Figure 2, B and C, respectively).

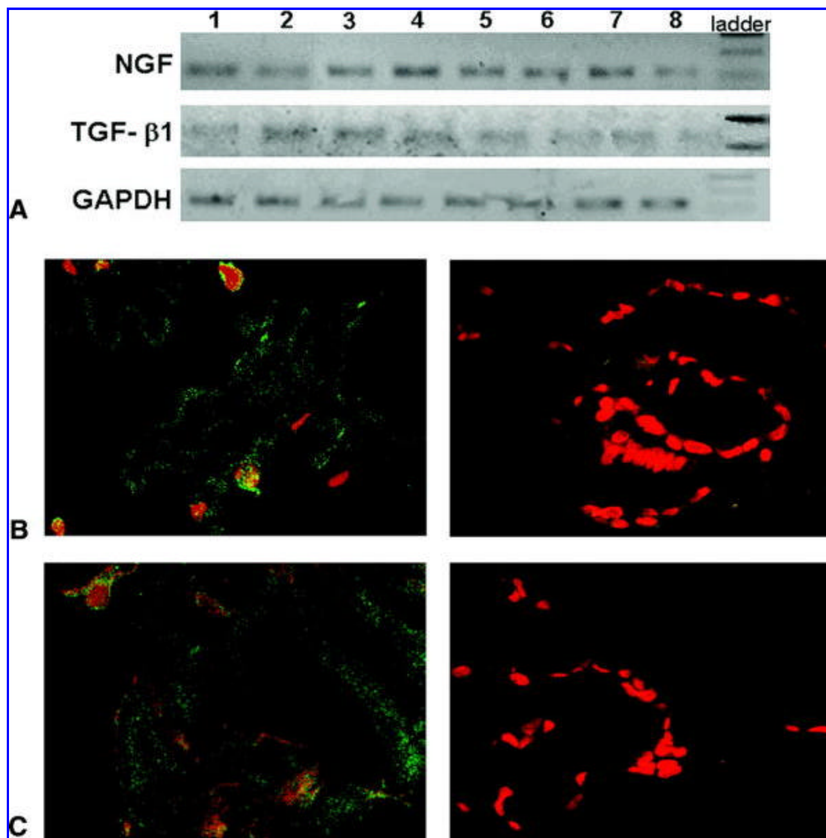

Fig. 2. Idiopathic ERMs express NGF and TGF-[beta]1. **A,** Semiquantitative RT-PCR: from upper to lower, agarose gels specific for NGF (120 bps), TGF- [beta]1 (110 bps), and GAPDH (100 bps). Data were normalized to GAPDH expression. **B, C,** Confocal microscope analysis of an ERM section showing respectively the expression of NGF (left panel; Cy2/green) and TGF -[beta]1 (left panel; Cy2/green). Nuclei were counterstained with propidium iodide (red). Right panels show isotype-control sections. Magnification ×200.

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trkA^{NGFR} and p75^{NTR} were detectable at both the biochemical (Figure 3A and B, respectively) and molecular levels (Figure 3D). In particular, confocal analysis showed that [alpha]-SMA/trkA^{NGFR} were coexpressed (Fig. 3A), while [alpha]-SMA/p75^{NTR} were colocalized (Figure 3B) in ERMs. trkA^{NGFR} and p75 ^{NTR} were colocalized in the same samples (Figure 3C).

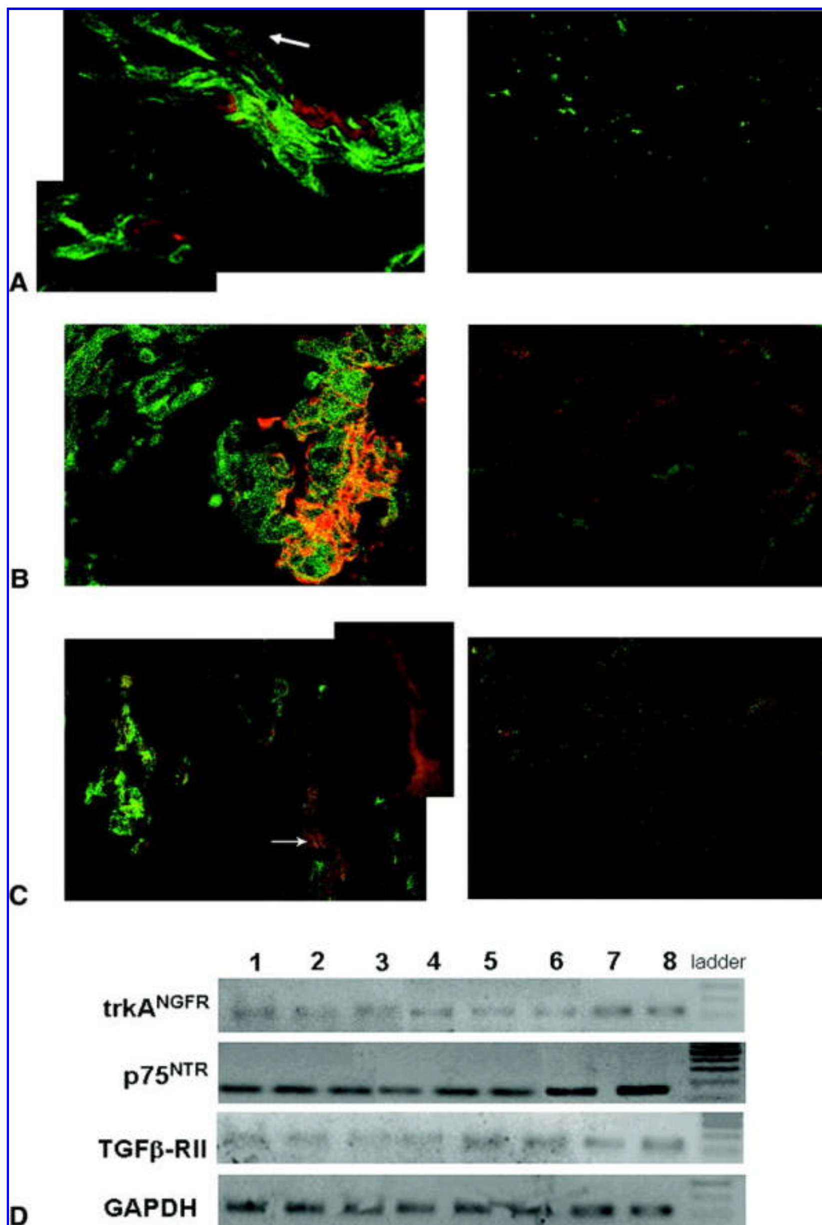

Fig. 3. Idiopathic ERMs express trkA^{NGFR}, p75^{NTR}, and TGF-[beta]RII. **A,** Confocal analysis (merge) showing that myoFBs express [alpha]-SMA and trkA^{NGFR} (left panel; Cy2/green-Cy3/red). [alpha]-SMA and trkA^{NGFR} coexpressed in some cellular compartments (arrow). In the frame, a high magnification stained cell is depicted. **B,** Confocal analysis (merge) shows that myoFBs express [alpha]-SMA and p75^{NTR} (left panel; Cy2/ green-Cy3/red). [alpha]-SMA and p75^{NTR} colocalized in some cellular compartments. **C,** Confocal analysis (merge) showing that p75^{NTR} and trkA^{NGFR} (left panel; Cy2/green-Cy3/red) are colocalized in some cellular compartments. In the frame, a high magnification stained cell is depicted. Right panels are isotype-control sections. Magnification ×200. **D,** Semiquantitative RT-PCR: from upper to lower, agarose gels specific for trkA^{NGFR} (102 bps); p75^{NTR} (147 bps); TGF-[beta]RII (121 bps); GAPDH (100 bps). Data were normalized to GAPDH expression.

TGF-[beta]RII mRNA was also expressed in ERMs (Figure 3D).

Vitreous From Patients With ERMs Showed Changes in NGF/trkA^{NGFR}-p75^{NTR} and TGF-[beta]1/TGF-[beta]RII Levels

ELISA evaluation of vitreous supernatants showed a significant decrease of NGF protein (101.29 ± 10.90 pg/mL versus 132.27 ± 26.18 pg/mL; $P < 0.05$; Figure 4A) and a nonsignificant increase of TGF-[beta]1 protein (985.00 ± 44.60 pg/mL versus 891.62 ± 11.23 pg/mL; $P > 0.05$; Figure 4B), as compared to controls.

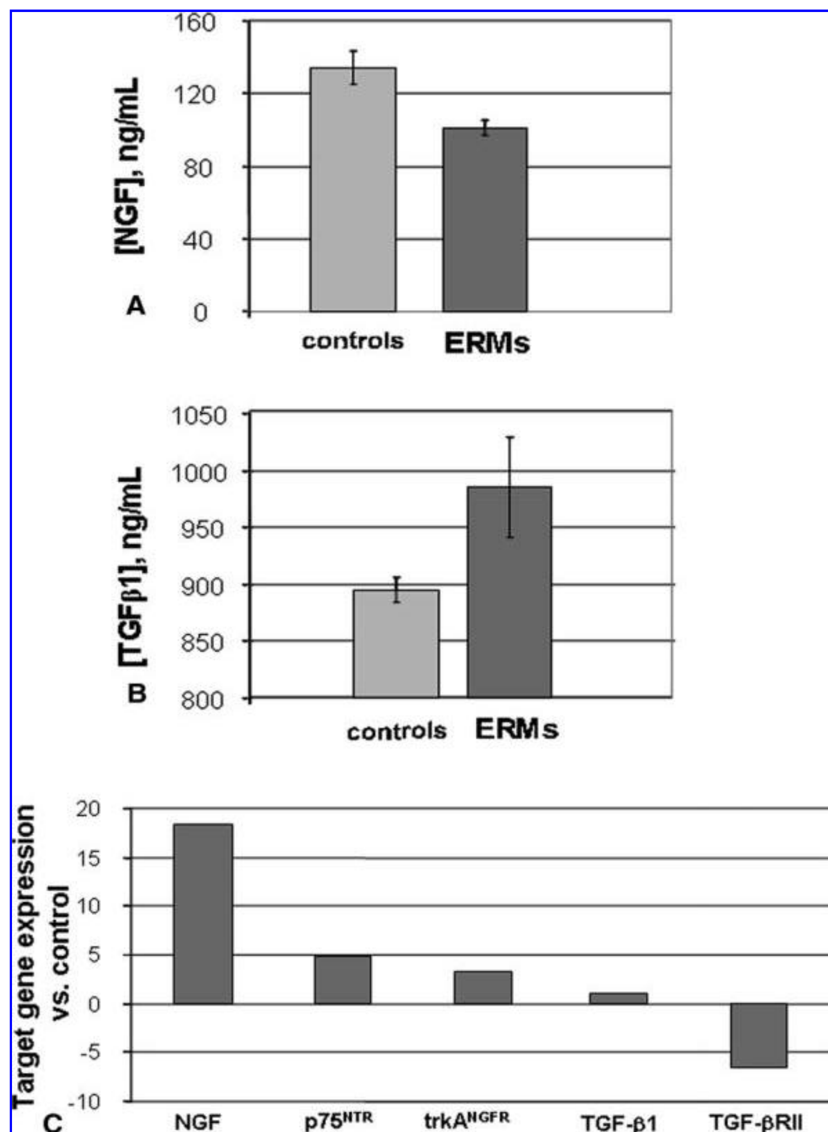

Fig. 4. NGF, trkA^{NGFR}, p75^{NTR}, TGF-[beta]1, and TGF-[beta]RII levels in the vitreous of patients with ERMs. **A, B,** NGF and TGF-[beta]1 ELISA assay in the vitreous of patients with ERMs compared to vitreous controls. Data are shown as mean right eye OD ± SEM. **C,** Relative real-time PCR showed an increase of NGF mRNA, trkA^{NGFR} mRNA, p75^{NTR} mRNA, and TGF-[beta]1 mRNA expression and a decrease of TGF-[beta]RII mRNA expression in the vitreous of patients with ERMs when compared to vitreous controls. Data were normalized to GAPDH expression, and presented as increase or decrease in target gene expression ± SEM with respect to vitreous controls (REST-ANOVA).

As shown in Figure 4C, vitreous cells showed a marked increase in NGF mRNA expression (18.46-fold increase; $P < 0.05$) while only a slight increase was observed for TGF-[beta]1 mRNA (1.05-fold increase, $P < 0.05$), when compared to controls. In addition, p75^{NTR} and trkA^{NGFR} mRNA were also significantly increased (4.8 and 3.3 target gene increase respectively, $P < 0.05$), while TGF-[beta]RII mRNA was significantly decreased (6.5 target gene decrease; $P < 0.05$), as compared to controls.

Discussion

Results from the present study demonstrate that 1) a consistent number of [alpha]-SMA positive myoFBs and a significant presence of NGF and TGF-[beta]1 characterize idiopathic ERMs; 2) trkA^{NGFR} and p75^{NTR} are specifically expressed by myoFBs in ERMs; and 3) both NGF and TGF-[beta]1 are impaired in the humor vitreous of patients with ERMs.

ERMs are classified as an idiopathic or secondary process and consist of fibroproliferative tissue characterized by cell-mediated contraction.[1–4] It is known that mechanisms other than defects in the internal limiting membrane might be responsible for transretinal cell migration, proliferation, and in situ differentiation.[11,13] Glial cells and myoFBs appear to be actively involved in the process.[5–8] Several myoFBs, detected by [alpha]-SMA expression at both biochemical and molecular levels, have been previously identified in secondary but not idiopathic ERMs.[20,21] The presence of myoFBs indicates that these cells, together with glial cells, macrophages, and RPE cells, might be involved in the process of idiopathic ERM growth. Glial cells and particularly FBs are a possible source of myoFBs in ERMs,[5,8] and the local increase of several growth factors and cytokines might drive this process.[11,13] Previous studies showed the role of several growth factors in modulating FB activities and their differentiation into myoFBs. [13–17] In particular, TGF-[beta]1, the main pro-fibrogenic factor, and NGF, involved in the healing and fibrotic process, are the most important stimuli for the induction of myoFBs and contractile actions.[14,17,19,20]

Although TGF-[beta]1 has been extensively studied in both idiopathic and secondary ERMs, scarce and conflicting data are available regarding NGF.[13,25] Only one study by Harada et al demonstrated the expression of trkA^{NGFR} mRNA in 1/7 and p75^{NTR}mRNA in 2/13 idiopathic ERMs.[25] In our study, NGF mRNA, trkA^{NGFR}/p75^{NTR} mRNA and protein, as well as TGF-[beta]1 were detected in all idiopathic ERMs. These apparently conflicting results might be due to 1) the different PCR technique used or 2) different stages of the disease, an unknown parameter since Harada and coworkers did not report the clinical features of their patients.[25]

If NGF drives myoFB formation, trkA^{NGFR} and p75^{NTR} would play an active role on [alpha]-SMA positive cells. The observation that myoFBs expressed both NGF receptors clearly indicates an autocrine/paracrine NGF effect, which, together with TGF-[beta]1, might modulate ERM cell function. The observation of TGF-[beta]RII [13] and trkA^{NGFR}/p75^{NTR} expression on [alpha]-SMA positive cells populating idiopathic ERMs clearly suggests that myoFBs are targets of TGF-[beta]1, NGF, or both.[13]

The levels of cytokines (IL-6) and growth factors (TGF-[beta]1, PDGF) in the vitreous are known to correlate with the clinical severity of ERM pathologies. 26 However, the influence of other factors such as NGF, released in the humor vitreous, produced by macrophages/hyalocytes 32-34 or released by ERMs themselves, should be taken into consideration.

According to the hypothesis that myoFBs can respond to NGF, 14,19 we quantified NGF in vitreous samples belonging to the same patients subjected to removal of ERMs. Interestingly, in the vitreous of patients with ERMs NGF mRNA was increased when compared to donors, while NGF protein was significantly decreased. This discrepancy may be explained considering the well-established pleiotropic activity of NGF. 14 Indeed, NGF might derive from different cell types populating ERM 5 which are able to increase NGF mRNA expression following cell-to-cell and/or mediator-cell signaling, during the inflammatory reactions and/or tissue repair. 20 In turn, by overexpressing NGF receptors, these cells can respond to NGF protein in a paracrine and/or autocrine fashion. The observation the increase in $trkA^{NGFR}/p75^{NTR}$ expression in vitreous cells, a finding that is known to occur in repairing/fibrotic mechanisms, 14 is consistent with this hypothesis.

NGF might also stimulate $trkA^{NGFR}$ and $p75^{NTR}$ expression to extend its activity, 24 with an increase in NGF utilization by target cells leading to the observed NGF protein decrease in the vitreous. Finally, the balance between production and utilization drives the NGF protein levels in vitreous. A similar phenomenon has been described in other ocular disorders and in vitro studies. 15 Nevertheless, we cannot exclude that vitreous cells (macrophages and hyalocytes)32-34 might also use NGF for growth and survival through paracrine/autocrine pathways.

Of particular interest is the finding that $p75^{NTR}$ is increased in both ERMs and related vitreous, because of its widely documented capacity of inducing apoptosis.35 In agreement with previous in vitro studies, NGF might modulate remodeling disorders by activating FBs but also by making myoFBs susceptible to apoptosis. 28 Impaired repair and excess of fibrosis might be the result of a complex interaction of several growth factors including NGF and TGF-[beta]1. 13

As demonstrated in earlier studies, TGF-[beta]1 was detected in ERMs. 13,25 We observed an increase in TGF-[beta]1mRNA and protein in the vitreous of patients with ERMs when compared to controls. In contrast, TGF-[beta]RII mRNA expression was decreased in vitreous cells. This finding might suggest a reduced TGF-[beta]1 utilization by vitreous cells, an explanation supported by the increased TGF-[beta]1 protein detected by ELISA.

In conclusion, our data open new perspectives on the pathogenic mechanisms of ERMs, prompting further investigation aimed at identifying the modulating roles of TGF-[beta]1 and NGF, with the ultimate goal of identifying a pharmacological means of treating ERMs.

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Key words: [alpha]-SMA; epiretinal membrane; myofibroblast; NGF; $p75^{NTR}$; TGF-[beta]1; $trkA^{NGFR}$

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Nerve growth factor has a modulatory role on human primary fibroblast cultures derived from vernal keratoconjunctivitis-affected conjunctiva

Alessandra Micera,[1,2] Alessandro Lambiase,[1] Barbara Stampachiacchiere,[1,2] Roberto Sgrulletta,[1] Eduardo Maria Normando,[1] Sergio Bonini,[3] Stefano Bonini[1]

[1]CIR Laboratory of Ophthalmology, University Campus Bio-medico; [2]IRCCS-G.B. Bietti Eye Foundation; [3]Second University of Naples & Institute of Neurobiology and Molecular Medicine, National Research Council (INMM-CNR), Rome, Italy

Purpose: To evaluate the role of nerve growth factor (NGF) in remodeling processes of vernal keratoconjunctivitis (VKC). VKC is a chronic inflammatory disorder of the conjunctiva and is characterized by marked tissue remodeling. NGF, a pleiotrophic factor with documented profibrogenic activities, is produced by inflammatory and structural cells populating the VKC conjunctiva and is increased in the serum and tears of VKC patients.
Methods: Primary cultures of VKC-derived fibroblasts (VKC-FBs) were exposed to increasing NGF concentrations (1-500 ng/ml) to evaluate and compare the expression of α-smooth muscle actin (αSMA, a defining myofibroblast marker), collagens (types I and IV), and metalloproteinases and tissue inhibitors (MMP9/TIMP1, MMP2/TIMP2) at the biochemical as well as molecular levels.
Results: Endogenous NGF was increased in the VKC-FB supernatant, as compared to healthy-FB supernatant. VKC-FBs expressed αSMA and increased types I and IV collagens. VKC-FBs, and in particular all αSMA positive cells, expressed both $trkA^{NGFR}$ and $p75^{NTR}$, while healthy-FBs only expressed $trkA^{NGFR}$. Exogenous NGF did not change αSMA expression, while αSMA expression was enhanced by specific neutralization of $p75^{NTR}$. NGF (10 ng/ml) exposure significantly decreased type I collagen expression, without affecting type IV collagen, and increased MMP9mRNA and protein.
Conclusions: The autocrine modulation of differentiation and response of VKC-FBs to NGF exposure with downregulation of type I collagen and upregulation of MMP9 expression supports a relevant role for NGF in tissue remodeling of VKC.

Vernal keratoconjunctivitis (VKC) is a severe chronic inflammatory disease of the conjunctiva. The disease is characterized by an allergic phenotype with infiltration of lymphocytes (mostly Th2), eosinophils, and mast cells [1]. In VKC, Th2 inflammation is associated with marked tissue remodeling as shown by fibroblast (FBs) activation, epithelial growth, subepithelial fibrosis, and extracellular matrix (ECM) deposition, resulting in giant papillae formation [2]. ECM analysis has shown a substantial increase in total collagen (procollagen and types I, III, and IV collagen), with an altered collagen ratio, both in tears and conjunctiva [3].Increased ECM is referred to increased expression of cytokines and growth factors, from either Th2-type and other inflammatory cells, which are known to stimulate resident/activated FBs to overproduce ECM [4]. Overt production and deposition of ECM eventually results from an unbalance between collagen production and Matrix Metalloproteinases (MMP1, MMP2 and MMP9) activities [1,3].

A variety of inflammatory- and stromal-derived cytokines, such as TNF-α, IL-1, IL-4, IL-13, and IL-6, and growth factors, such as transforming growth factor β1 (TGFβ1) have been reported to cause chronic inflammation, structural changes, tissue remodeling, as well as fibrosis [5,6]. All these factors are increased in the peripheral blood, tears, and conjunctiva of patients with VKC [2].

To date, TGFβ1 represents the main profibrogenic factor responsible for the imbalance in ECM metabolism as well as for the chemoattraction and survival of FBs and myoFBs. Interestingly, nerve growth factor (NGF) has been recently prospected as another factor playing a role in stromal-epithelial interaction during wound-healing and tissue repair processes [7,8]. Certainly, NGF is significantly increased in both serum and conjunctiva of patients with VKC [9].

We previously reported a profibrogenic NGF effect on in vitro primary cultures of healthy conjunctival FBs (healthy-FBs) [8]. Since nothing is known about the possible role of NGF (increased in VKC-affected conjunctiva) on primary cultures of VKC conjunctival FBs (VKC-FBs), we exposed these cells to increasing NGF concentrations. In addition, some in vitro parameters of tissue remodeling were evaluated.

METHODS

Reagents: Unless otherwise noted, sterile tissue culture plastic-ware and analytical grade reagents were from NUNC (Roskilde, Denmark), SERVA (Weidelberg, Germany), ICN (Costa Mesa, CA), Euroclone (Milan, Italy), and Invitrogen-Gibco (Paisley, UK).

Cell culture and experimental procedure: FBs were isolated from the upper tarsal VKC (n=3; 3M, range 11-15, mean

Correspondence to: Stefano Bonini, MD, Department of Ophthalmology, University of Rome Campus Bio-Medico, Via Emilio Longoni 83, 00155 Rome, Italy; Phone: (39) 06-22541342; FAX: (39) 06-22541342; email: s.bonini@unicampus.it

age 12.67±2.08) and sex/age matched healthy (n=3) conjunctiva. Informed consent was given by the parents of each patient and the approval of the Intramural Ethic Committee was granted, in conformity to the Declaration of Helsinki. The diagnosis of active VKC was based on history, clinical examination and the presence of eosinophils in conjunctival biopsy. Clinical scores (0-3: 0, absent; 1, weak; 2, mild; 3, severe) for each ocular symptom (itching, tearing, photophobia and foreign body sensation) and each sign (conjunctival hyperemia, mucous discharge, papillae and corneal epithelial erosion) were assigned at the time of examination. Total symptom (range 0-3) and sign scores (0-12) were calculated showing: total symptom score of 7.33±4.51 and total sign score of 8.67±3.05. The three patients showing lid-ptosis were submitted to surgical removal of the papillae that were processed in this study.

Biopsies were put as explants in 24-well plates and left to attach for 10 min, before adding medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells outgrowing from biopsies (37 °C, 5% CO_2 in air, by 1 week), were quickly trypsinized (0.2% trypsin/0.025% EDTA), assessed for the absence of epithelial cell contaminants (cytokeratin 19, 1:100; Dako Corp., Carpinteria, CA) and screened as mycoplasm free cultures (otherwise treated for 1 generation with 5 µg/ml Mycoplasm Removal Agent; ICN). FBs were sub-cultured (T-21/T-75 cm^2 flasks) and used for the experiments (3rd to 4th passage) after 3 h of serum starvation (synchronization and reduction of autophosphorylation) [10].

For the specific experiments, confluent FBs were treated with increasing NGF concentrations (murine βNGF; 1-500 ng/ml; produced according to a standardized protocol [11]) in minimal DMEM (0.5% FBS) [8]. Biochemical and molecular evaluations were performed 2 days after stimulation. For blocking experiments, serum-starved FBs were preincubated for 1 h with 500 ng/ml αNGF (R&D Systems, Minneapolis, MN) or with 100 ng/ml $\alpha trkA^{NGFR}$ (Calbiochem, La Jolla, CA) or 100 ng/ml $\alpha p75^{NTR}$ (Calbiochem) specific neutralizing antibodies, before the addition of NGF alone (untreated FBs) or in the same blocking reagents (pretreated FBs), for two days. Whenever required and according to literature [6,12], human TGFβ1 (1-10 ng/ml, R&D) was used as positive control in some experiments.

Confocal microscopy: Confluent monolayers (on sterilized round glasses, EMS, Hatfield, PA) were washed in Hank's Balanced Sodium Salt (HBSS), fixed in 2% ρ-formaldehyde (PFA) - 0.1 M Phosphate Buffer (PB), rinsed in 10 mM PB - 137 mM NaCl (phosphate buffered saline, PBS), quenched in 50 mM NH_4Cl-PBS, permeabilized in 0.5% Triton X-100 PBS (TX-PBS) and finally blocked in 0.8% bovine serum albumin (BSA)-PBS. Monolayers were then probed with the following antibodies in 0.05% Tween20-PBS (TW-PBS): rabbit anti-human $trkA^{NGFR}$ antibody (2 µg/ml; Santa Cruz Biotech, Santa Cruz, CA); goat anti-human $p75^{NTR}$ antibody (2 µg/ml; Santa Cruz) and mouse anti-human αSMA antibodies (1/50; Novocastra, Milan, Italy). Specific binding of the primary antibody was detected using Cy2 or Cy3 conjugated $F(ab)_2$ antibodies in TW-PBS (Jackson Laboratories, West Grove, PA). Control immune-staining was performed by substituting primary antibodies with control irrelevant IgG (Vector Laboratories, Burlingame, CA). Glasses were mounted on slides using an anti-fade medium (Vectashield, Vector) and viewed with a confocal inverted microscope (E2000U; Nikon, Tokyo, Japan). Images and brightness/contrast levels were captured/evaluated using the C1 software (Nikon) and the Adobe Photoshop 7.0 program (Adobe Systems Inc., San Jose, CA), respectively.

Relative real-time RT-PCR: Total RNA ($1x10^6$ cells) was extracted in OMNIzol (Euroclone), DNaseI treated (AB1709; Ambion Inc., Austin, TX), quantified ($\lambda_{260}/\lambda_{280}$ >1.8) and checked for RNA integrity. Three µg total RNA were reverse transcripted (Mu-MLV; Finnzyme, Milan, Italy) in a PTC-100 programmable thermocycler (MJ Research, Watertown, MA). cDNAs (3 µl for target gene and 1 µl for referring gene) were amplified using the Opticon2 real time termocycler (MJ Research) in a 20 µl final volume of SYBR Green PCR mixture (Applied Biosystems, Foster City, CA). The temperature profile included initial 95 °C for 15 min incubation, followed by 35-47 cycles of denaturation at 95 °C for 30 s, annealing at 55-60 °C for 25 s (see previous paragraph for specific Ta), elongation at 72 °C for 30 s, fluorescence monitoring at 60-90 °C, 0.01 °C for 0.3 s, and further incubation at 72 °C for 5 min. PCR amplification primer pairs were received from Genbank or Primer 3 (MWG Biotech, Ebersberg, Germany), were as follows: NGF (forward: CTG GCC ACA CTG AGG TGC AT; reverse: TCC TGC AGG GAC ATT GCT CTC; 120 bp; 53 °C Ta; Genbank BC011123); $trkA^{NGFR}$ (forward: CAT CGT GAA GAG TGG TCT CCG; reverse: GAG AGA GAC TCC AGA GCG TTG AA; 102 bp; 58 °C Ta; Genbank M23102); $p75^{NTR}$ (forward: CCT ACG GCT ACT ACC AGG ATG AG; reverse: TGG CCT CGT CGG AAT ACG; 147 bp; 57 °C Ta; Genbank AF187064); αSMA (forward: GAA GGA GAT CAC GGC CCT A; reverse: ACA TCT GCT GGA AGG TGG AC; 125 bp; 60 °C Ta; Genbank BC017554); MMP-9 (forward: CAG TCC ACC CTT GTG CTC TTC C; reverse; GCC ACC CGA GTG TAA CCA TAG C; 113 bp; 60 °C Ta; Genbank BC006093) and the referring GAPDH gene (forward: GAA GGG GTC ATT GAT GGC AAC; reverse: GGG AAG GTG AAG GTC GGA GTC; 100 bp; 53 °C Ta; Genbank BC013310). Product specificity was assessed by melting curve analysis of each sample carried out in duplicate as well as by gel size-fractioning. C_t values normalized samples showing good melting curves were used for statistical analysis. Differences in PCR product expression were evaluated by REST© software [13].

Proliferation assays: The effect of increasing NGF concentration on VKC-FB proliferation was investigated by counting the cells, after brief enzymatic digestion, using the trypan blue exclusion test. The expression of the nuclear proliferating factor ki67, recognizing all the cell cycles except G0 [14], was investigated on previously fixed monolayers stained with rabbit antihuman ki67 antibody (1:1000, Santa Cruz) and developed by fluorescent ABC technique (Vector).

Nerve growth factor ELISA: To evaluate NGF in the su-

pernatants of VKC and healthy FBs, we carried out a a two-site NGF ELISA (sensitivity=0.5 pg/ml) [15]. In brief, 96-well Maxisorp ELISA plates were precoated with monoclonal antihuman βNGF antibodies (0.4 μg/ml; MAB256, RαD). Both standards (0.15 pg/ml to 1 ng/ml βNGF; Alomone, Jerusalem, Israel) and samples (1/4) were incubated at 4 °C for 18 h. ELISA was developed by using polyclonal biotinylated antihuman ηNGF antibodies (0.15 μg/ml, 500-P85Bt; Peprotech, Milan, Italy), HRP-streptavidin (1/300; DY998, RαD) and the ready-to-use TMB substrate (Zymed, San Francisco, CA). Optical density (OD) was measured at $\lambda_{450-550}$ by a microplate ELISA reader (Sunrise, Tecan Systems, Inc., San Jose, CA). The biological activity of FB-derived NGF was tested separately by using a standardized PC12 bioassay [16]. Protein normalization was achieved by a Bio-Rad protein assay, using BSA equivalent as a standard (Bio-Rad Laboratories, Inc., Hercules, CA).

Cell surface ELISA: To investigate the effect of NGF on αSMA expression by VKC-FBs, an ELISA was carried out on monolayers [17]. Briefly, FBs ($5x10^5$/0.20 ml) were seeded on 96-well plates. Once the cells reached confluence, they were incubated with increasing NGF concentrations. After culturing, plates were washed in HBSS containing 0.1% $CaCl_2$, PFA post-fixed, quenched for endogenous peroxidases (0.3% H_2O_2 in PBS for 15 min), permeabilized in TX-PBS, and dehydrated with 95% ethanol before the assay. The plates were subsequently processed for quantification of αSMA (1/500, Novocastra), types I and type IV collagen (1/1000; Santa Cruz) proteins, using monoclonal and polyclonal specific antibodies diluted in TW-PBS. Biotin-conjugated antimouse or antigoat antibodies and HRP-streptavidin (1/10000 and 1/7000, respectively; Zymed) specific binding were developed with a ready-to-use TMB and quantified using an ELISA reader.

Western blot analysis: Total proteins were extracted by 30 min incubation with cold lysis-buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 7 μg/ml aprotinin, and 1 mM PMSF). Equivalent protein amounts (40-80 μg; Bio-Rad protein assay) were boiled for 5 min under reducing conditions and fractionated on a 4% stacking/6-15% gradient resolving gel at 160 V/60 min (Miniprotean3 apparatus, Bio-Rad). Electrophoresed proteins were transferred to Hy-bond membranes using a semi-dry blotting apparatus (12 V/45 min, Bio-Rad). A molecular weight marker (6-210 kDa; SERVA) was run in parallel. Transferred proteins (Ponceau S staining) were probed at 4 °C for 18 h with primary antibodies diluted in TW-PBS (αSMA, types I and type IV collagen: 0.2 μg/ml; MMP9: 0.4 μg/ml), labeled with secondary POD-conjugated specific antibodies (1/7000, 90 min; Jackson), and developed by ECL technique (SuperSignal West Pico Trial; Pierce, Rockford, IL) in a high performance Kodak imager station (Kodak 550, Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY). Bands were digitally captured using the 1D Kodak Image Analysis Software, subjected to densitometric analysis, and processed in Adobe Photoshop 7.0. Membranes were stripped at 56 °C for 45 min in 1 M Tris-HCl containing 2% SDS and 1.25 mM β-mercaptoethanol and reprobed with GAPDH antibodies (0.2 μg/ml; Abcam, Cambridge, UK), to verify equal protein loading.

SDS-PAGE zymography: After culturing, the conditioned media were collected and clarified by centrifugation. MMP activity was analyzed by zymography, using a procedure described in reference [18]. Briefly, 50 μl conditioned media were mixed with SDS sample buffer, without β-ME, and heated for 37 °C/30 min. Normalized samples (30 μg/lane; Bio-Rad protein assay) underwent standardized electrophoresis in 10% SDS-PAGE containing 0.1% gelatin (Bio-Rad). Molecular weight markers (6-210 kDa), recombinant human latent MMP9 (92 kDa), and active MMP9 (83 kDa; Calbiochem) were loaded as positive controls. The gel was washed in 2.5% TX-PBS to remove SDS and renature the proteins, incubated at 37 °C for 48 h in an activation buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$; pH 7.5), rinsed in ddw, and finally stained for 60 min with 0.25% Coomassie brilliant blue R250 in 40% isopropanol. Gelatinolytic activity was identified as clear bands on a uniform blue background following destaining in 7% acetic acid, indicating the area where gelatin was digested.

Statistics: All experiments were done three times, with each point carried out in duplicate. Experimental results are expressed as mean±SEM. Parametric ANOVA followed by Tukey-Kramer post hoc was employed to analyze the data [19], using the statistical package StatView II for PC (Abacus Concepts Inc., Barkley, CA). A probability of less than or equal to 0.05 was considered to be statistically significant.

RESULTS

Vernal keratoconjunctivitis-derived fibroblasts express nerve growth factor, trkA^{NGFR}, p75^{NTR}, and αSMA: Increased NGF levels were detected by specific ELISA in the conditioned media of VKC-FBs and compared to healthy-FBs (563.33±35.12 pg/ml versus 445.67±10.59 pg/ml; 26.51% increase; p=0.005). This data is in accordance with the NGF mRNA upregulation observed in VKC-FBs (Figure 1A). RT-PCR and confocal analysis revealed that VKC-FBs expressed both trkA^{NGFR} and p75^{NTR}. In contrast, healthy FBs only express trkA^{NGFR}, as previously reported [8] (Figure 1A,B). A slight downregulation of trkAmRNA expression was found in VKC-FBs, in comparison to healthy ones ($p<0.05$). As detected by cs-ELISA, a significant increase of αSMA expression was observed in VKC-FBs (0.378±0.027 OD VKC-FBs versus 0.232±0.046 OD healthy-FBs; a 1.62-protein increase [$p<0.01$]). VKC-FBs expressed αSMA protein in association with p75^{NTR} (Figure 1C). No proliferation was observed in the presence of increasing NGF concentrations (data not shown).

Nerve growth factor does not modulate αSMA expression: VKC-FBs were exposed to increasing NGF concentrations (1-500 ng/ml) in order to investigate whether NGF was able to influence αSMA expression. At both cs-ELISA and western blot analysis, NGF was not able to influence αSMA expression in VKC-FBs (Figure 2; dark bars [$p>0.05$]). NGF stimulation did not influence αSMA expression after preincubations with neutralizing trkA^{NGFR} antibodies, (data not shown). In contrast, preincubation with neutralizing p75^{NTR} antibodies followed by increasing NGF concentrations resulted in an in-

crease of αSMA expression (Figure 2; light bars [$p<0.05$]). The pattern of αSMA expression resembled those of NGF-treated healthy FBs [8].

Nerve growth factor modulates types I and IV collagen expression: In VKC-FBs, the high amount of αSMA was found associated with a high rate of types I and IV collagen (rate; 7.78: type I, 1.323±0.248 and type IV, 0.170±0.004), as compared to healthy FBs (type I, 0.993±0.001 and type IV, 0.077±0.014; $p<0.05$; see also Table 1). After NGF exposure, the expression of type I collagen by VKC-FBs was significantly decreased at all the concentration levels tested (Figure 3A). The maximum effect was observed at 10 ng/ml (1.33-target gene decrease, $p<0.05$), reaching a level almost comparable to those of healthy-FBs (Figure 3A; light bar). By contrast, the expression of type IV collagen was not changed at any NGF concentration level (Figure 3B; light bars).

Nerve growth factor modulates MMP-9 expression and function: The expression and functional activities of MMP9, known to drive specifically collagen type IV cleavage [20], was investigated as a function of NGF stimulation in VKC-FBs. VKC-FBs were exposed to increasing NGF concentra-

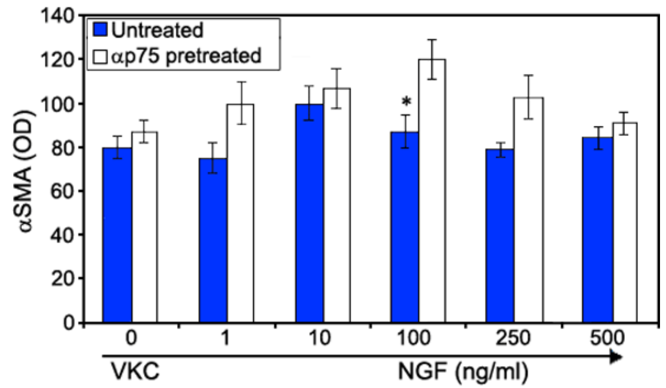

Figure 2. αSMA expression by vernal keratoconjunctivitis-derived fibroblasts. Increasing NGF concentrations did not influence αSMA expression by VKC-FBs (dark bar; $p>0.05$). αp75^{NTR} neutralization (1 μg/ml/30 min) significantly increased αSMA expression by NGF-treatment (light bar; $p<0.05$). αtrkA^{NGFR} neutralization did not influence αSMA expression by NGF treated VKC-FBs (data not shown). Representative data from three independent experiments, which results are shown as mean OD±SEM.

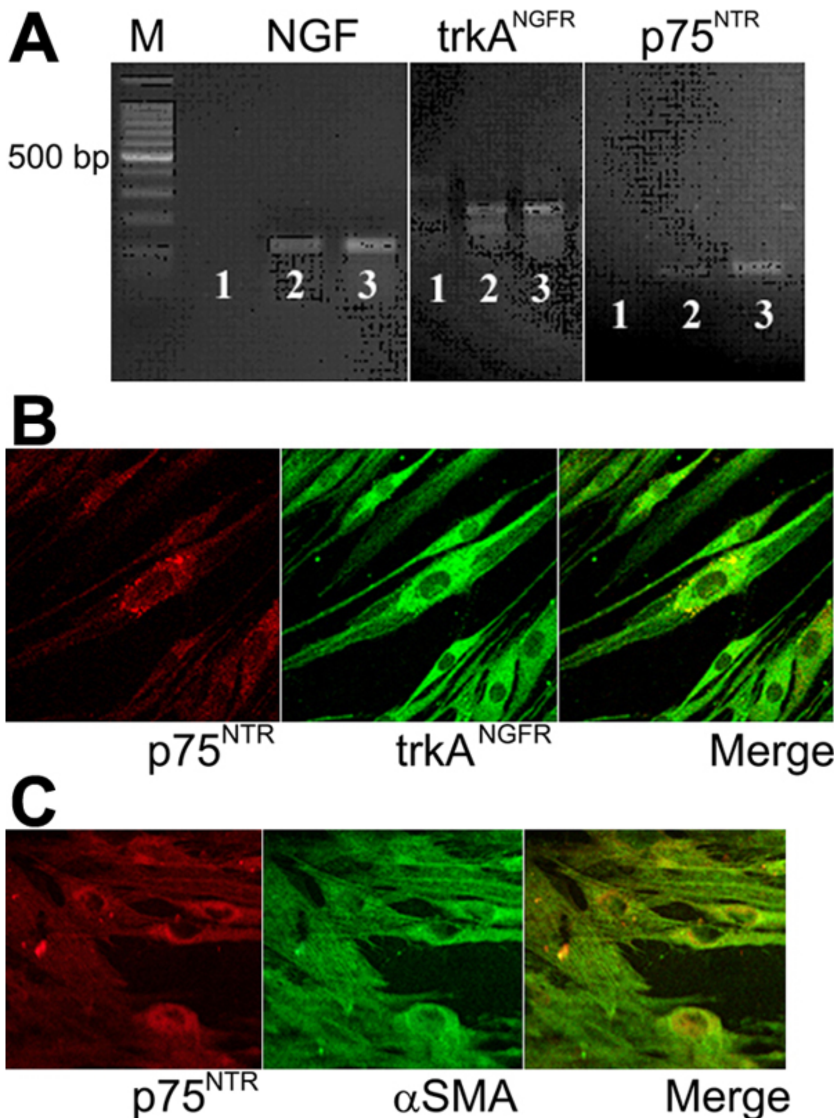

Figure 1. Nerve growth factor, trkA^{NGFR}, p75^{NTR} and αSMA expression by vernal keratoconjunctivitis-derived fibroblasts. **A**: Conventional RT-PCR. showing from left to right, nerve growth factor (NGF; 120 bp), trkA^{NGFR} (103 bp); p75^{NTR} (100 bp) amplicons. The lines are as follows: (1) -RT, (2) healthy-FBs, and (3) VKC-FBs. These are representative gels from three independent experiments where equal amounts of cDNA were amplified. **B**: Confocal microscopy on VKC-FBs showing, from left to right, that VKC-FBs express p75^{NTR} (Cy3, red) and trkA^{NGFR} (Cy2, green). p75^{NTR} and trkA^{NGFR} colocalized in some cellular compartments (merge; X600/oil immersion). **C**: Confocal microscopy on VKC-FBs showing, from left to right, that these cells express p75^{NTR} (Cy3, red) and αSMA (Cy2, green). p75^{NTR} and αSMA colocalized in some cellular compartments (merge; X600/oil immersion).

tions (1-100 ng/ml) and the expression of MMP9 protein and mRNA were evaluated and compared to untreated VKC-FBs. By western blot analysis, it was determined that NGF significantly increased MMP9 protein expression in VKC-FBs, as compared to healthy-FBs, in a dose-dependent fashion (Figure 4A). This specific MMP9 increase was associated with an increase of MMP9 activities, as shown by gelatinolytic bands, at the same concentrations tested (Figure 4B). In agreement with biochemical data, the molecular analysis showed that NGF was able to trigger the MMP9-mRNA expression in a dose-dependent fashion with the maximum expression at 10 ng/ml NGF (3.92-target gene increase, $p<0.05$; Figure 4C). Interestingly, the MMP9-mRNA increasing effect of 10 ng/ml NGF was found to be comparable to that of 1 ng/ml TGFβ1 (5.48±0.04 versus 7.49±0.09, respectively). According to the literature, MMP9 mRNA was found to be increased in VKC-FBs as compared to healthy-FBs (24.98-target gene increase; $p<0.05$ [3]). Biochemical and molecular characteristics of healthy and VKC-derived FBs are summarized in Table 1.

DISCUSSION

Our data demonstrated that primary cultures of VKC-FBs express both $trkA^{NGFR}$ and $p75^{NTR}$ receptors and produce high levels of NGF, types I and IV collagens, and MMP9. Moreover, VKC-FBs are mainly represented by myoFBs, since a consistent proportion of VKC-FBs were αSMA positive.

Various Th2-derived cytokines and growth factors are increased in blood, tears, and conjunctiva from VKC patients. These profibrotic factors were found able to drive the cross-talk between structural (epithelium and FBs) and inflammatory (lymphocytes, eosinophils, and mast cells) cells. This cell-factor interaction contributes to the chronic inflammatory process, giant papillae formation and tissue remodeling, as observed in VKC [2,3,5,21-23]. Among these, TGFβ1 isoform remains the main pro-fibrogenic factor, being responsible for ECM ex-novo deposition, the inhibition of ECM degradation and the prolonged myoFB activity [24].

NGF is also increased in VKC blood and conjunctiva as a result of the activation of both structural (epithelium and FBs) and inflammatory cells (Th2 lymphocytes, mast cells, and eosinophils), during the active conjunctivitis [7,22]. Older and more recent data [8,22-24] indicate that NGF is a pleiotrophic factor participating to the control of inflammatory responses, tissue repair, fibrosis, and remodeling in different tissues. Primary cultures of healthy-FBs have been found to be modulated by NGF with relation to cell migration, differentiation, and contraction of a cell matrix [8].

Since stromal FBs represent the major target/effector cells involved in tissue remodeling [6,12,25] and since NGF activates in vitro healthy-FBs [8,26,27], we sough to evaluate the possible modulation of FBs isolated from conjunctiva of patients with VKC by NGF, hence no data are available in literature.

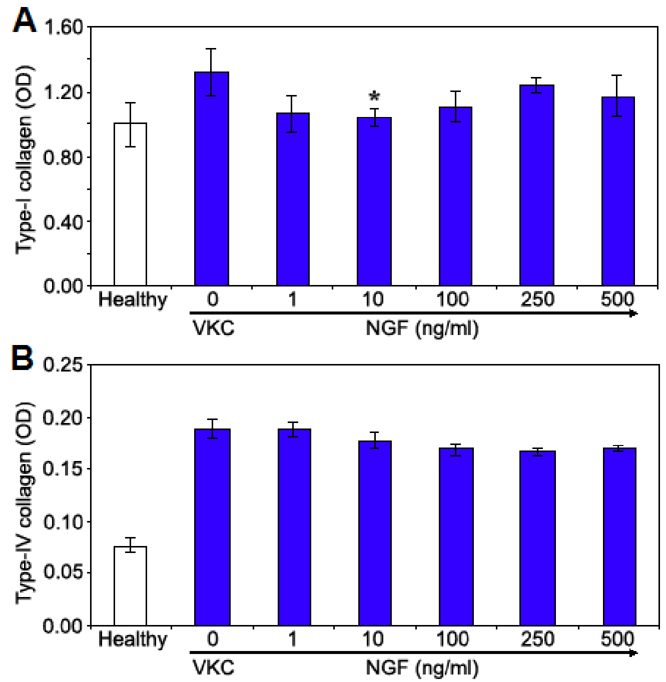

Figure 3. Nerve growth factor modulates type I and type IV collagen expression. VKC-FBs showed high types I and IV collagen production in comparison to healthy-FBs (rate: 7.78; $p<0.05$). Increasing NGF concentrations significantly decreased type I collagen expression (**A**), with a maximum at 10 ng/ml NGF (1.33 fold decrease; $p<0.05$). No effect was observed on type IV collagen expression (**B**). Representative data from three independent experiments are shown as mean OD±SEM.

Table 1. Biochemical and molecular description of healthy and vernal keratoconjunctivitis-derived fibroblasts

Experimental group	Type-I collagen*	Type-IV collagen*	αSMA**	MMP9**	TIMP1**
Healthy (n=3)	0.993±0.001	0.077±0.014	3.60±0.70	8.01±0.22	8.70±0.12
VKC (n=3)	1.323±0.248	0.170±0.004	2.24±1.67	6.08±0.78	12.82±0.98
Analysis	*+33%*	*+120%*	*+2.57*	*+3.81*	*-5.69*

Biochemical and molecular description of healthy and vernal keratoconjunctivitis-derived fibroblasts

In the present study, increased expression of NGF, associated with increased expression of $p75^{NTR}$ and αSMA, was detected in VKC-FBs as compared with conjunctival healthy-FBs. In addition, αSMA was found expressed in FBs showing light upregulation of $trkA^{NGFR}$ and considerable upregulation of $p75^{NTR}$, suggesting a specific role for NGF in VKC-FBs. In line with the effect of NGF in driving the differentiation of healthy FBs into myoFBs [8], we wondered whether NGF supplementation to VKC-FBs cultures would result in a further VKC-FB differentiation, evaluated as αSMA expression. Interestingly, NGF failed to further increase αSMA expression in VKC-FBs, unless $p75^{NTR}$ was blocked (see Figure 2; light bars). This data is of great interest, since it suggests two possible hypothesis: (1) NGF plays a differentiating effect through the specific and unique binding to $trkA^{NGFR}$ (NGF is a specific receptor), but not $p75^{NTR}$ (the pan-neurotrophin receptor); and (2) the specific expression of $p75^{NTR}$, by myoFB phenotype (otherwise absent in FBs), seems to play a switch-off effect in the further differentiating action of NGF, suggesting that NGF plays a modulatory rather than a exclusive stimulatory effect on the fibrotic process. The answer might lie in $trkA^{NGFR}/p75^{NTR}$ multifaceted functions [7,22].

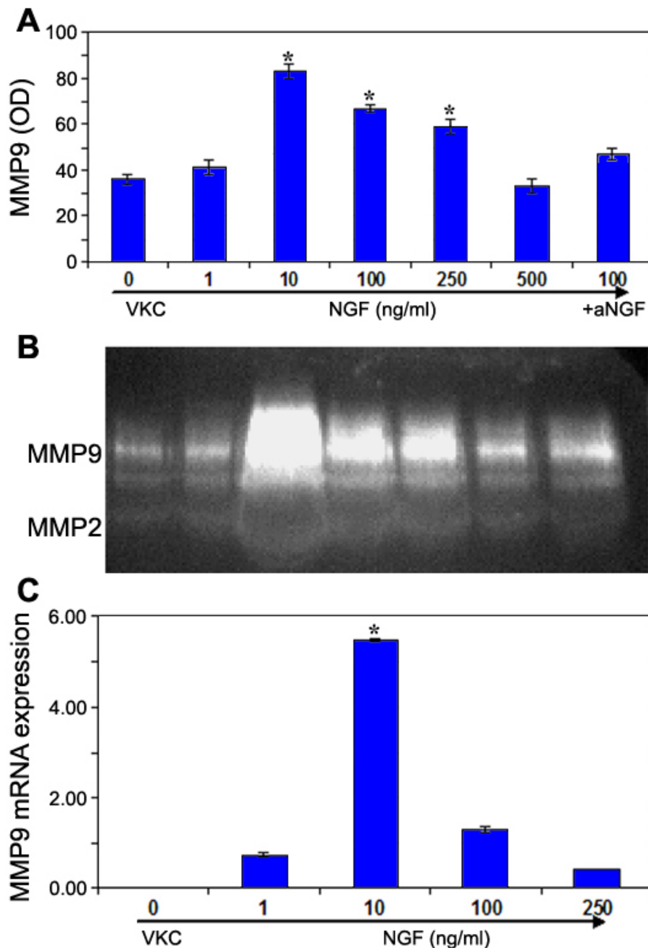

Figure 4. Nerve growth factor increases MMP9 expression and function by vernal keratoconjunctivitis-derived fibroblasts. Conditioned media were collected and processed as described in Methods. **A**: The histogram shows a significant increase of MMP9 protein expression in VKC-FBs treated with increasing NGF concentrations, according to the densitometric analysis (mean OD±SEM; $p<0.05$). Data were normalized to GAPDH expression and presented as fold increase with respect to untreated VKC-FBs. **B**: The functional activity in MMP9 was investigated by SDS-PAGE zymography. From left to right (1-6 lines): 0, 1, 10, 100, 250, 500 ng/ml NGF; line 7, αNGF+100 ng/ml NGF. Panel represents one of three independent gels that gave the same results. **C**: Relative real-time PCR showed a significant increase of MMP9 mRNA expression in VKC-FBs treated with different concentrations of NGF ($p<0.05$). Data were normalized to GAPDHmRNA expression and presented as fold increase [11] with respect to untreated VKC-FBs.

On the other side in VKC tissue remodeling process, NGF might modulate collagen production and might influence MMP2/MMP9 production/activity, as previously demonstrated for other growth factors [2,4]. ECM metabolism is heavily impaired in VKC, due to a substantial increase in total collagen deposition in the conjunctiva (mainly types I, III, and IV), and an increased release of MMP2/MMP9 in the tears [3,4]. In this study, the expression of type-I collagen decreased significantly after NGF exposure, rather quite specific given that this effect was not observed for type-IV collagen or with the addition of TGFβ1 (data not shown). In addition, NGF was able to induce specifically MMP9 expression/activity by VKC-FBs at both molecular, biochemical and functional levels. This effect was not observed in healthy-FBs, as previously reported [8].

Taken together, these data suggest a many-sided role of NGF in VKC tissue remodeling. NGF is increased in VKC blood and tarsal conjunctiva and likewise in tears [9]. In in vitro studies, NGF induces the differentiation of healthy conjunctival FBs into myoFBs, the main effector and target cells of fibrotic process. This differentiating effect was not observed in VKC-derived conjunctival FBs in the present study. Additionally, NGF induced a decrease in type I collagen and an increase in MMP9 expression by VKC-FBs with no specific effect on both MMP1 and MMP2 mRNA expression. It has been previously reported that both MMP2 and MMP9 degrade types IV and V collagen; MMP9 can also degrade types I and type III collagen [28,29]. One possible explanation for these data might be related to the well-known singular action of NGF due to changes in the balance between $trkA^{NGFR}/p75^{NTR}$ expression. In this study we demonstrated a role of $trkA^{NGFR}$ in the differentiating effect of NGF on FBs; however, the role of $p75^{NTR}$ on VKC-FB function seems complex and remains to be elucidated.

In VKC, various growth factors and Th2-cytokines are produced by inflammatory/stromal cells. In line with other studies, these factors have been proposed to modulate tissue remodeling in VKC. To summarize, our in vitro findings showing the NGF modulation of both type I collagen and MMP9, might propose NGF as an active contributor in VKC remodeling.

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Toll-like receptors and the eye

Alessandra Micera[a], Barbara Stampachiacchiere[a], Silvia Aronni[a], Myrna Serapiao dos Santos[a,b] and Alessandro Lambiase[a]

Purpose of review
This review will describe the structure, expression/distribution and functional activity of Toll-like receptors, in particular in the ocular structures. It will also discuss innate and adaptive immune responses, by exploring the possible modulation/regulation of innate and adaptive immunity by Toll-like receptors, in view of recent findings observed in the ocular surface.

Recent findings
Current knowledge indicates that Toll-like receptors represent essential elements in host defence against pathogens, a prerequisite to the induction of adaptive immune responses. The expression/distribution of Toll-like receptors in the healthy eye highlights the possible function of Toll-like receptors in both innate and adaptive responses during pathological conditions of the ocular surface.

Summary
Recent findings have greatly increased the knowledge of the possible role of Toll-like receptors in innate and adaptive immune responses. Toll-like receptors seem to play different roles in a wide range of activities of the immune system, and might represent an exclusive link between innate and adaptive responses under pathological conditions. Recent studies in ophthalmology have highlighted the role of Toll-like receptors in infections (keratitis) as well as in allergic states of the ocular surface. This review thus describes the relationship between Toll-like receptors and the main immune/structural cells taking part in inflammatory disorders. Understanding the complex mechanisms underlying Toll-like receptor localization and function will provide additional data that might help devise novel therapeutic approaches involving Toll-like receptors and their agonists, in an attempt to modulate the biased immune system.

Keywords
allergy, corneal keratitis, infections, ocular surface, Toll-like receptors, vernal keratoconjunctivitis

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[a]CIR Laboratory of Ophthalmology, University of Rome Campus Bio-Medico and G.B. Bietti Foundation, Rome, Italy and [b]Advanced Center of Ocular Surface, Vision Institute, Federal University of São Paulo, São Paulo, Brazil

Correspondence to Alessandro Lambiase, MD, Department of Ophthalmology, University of Rome 'Campus Bio-Medico', Via Emilio Longoni 83, 00155 Rome, Italy
Tel: +39 06 22541342; fax: +39 06 22541456; e-mail: a.lambiase@unicampus.it

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Abbreviations

DC	dendritic cell
ISS-ODN	immunostimulatory sequence oligodeoxynucleotides
NFκB	nuclear factor kappa B
RPE	retinal pigment epithelial
TLR	Toll-like receptor
VKC	vernal keratoconjunctivitis

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Introduction

Whereas the highly specific adaptive response requires time to produce immunoglobulins and cell-mediated immune reactions, the innate immune system, recognizing structurally-conserved microbial/viral products, allows a fast and proper immune response to limit or completely destroy invading pathogens [1,2]. Toll-like receptors (TLRs) play a crucial role by recognizing proteins or DNA/RNA sequences belonging to bacteria, protozoa, helminths, fungi and viruses [1]. Specific TLR activation results in the production of pro-inflammatory mediators and cytokines, driving the antimicrobial host response [3,4••,5]. The ability of TLRs to drive innate and adaptive immune responses resides in specific TLR expression by host immune-competent cells [dendritic cells (DCs) and scavenger cells], which are the first cell types to come into direct contact with pathogens from the outer environment [6].

Ten human TLRs have been characterized, and their differential expression has been shown in gastrointestinal, airway and urinary epithelia, in primary and secondary lymphoid organs, and, as recently described, in the eye.

In particular, the ocular surface is continuously exposed to pathogens, and therefore has to fight infection through mechanical protection as well as through mechanisms of humoral, cellular, adaptive, and innate immunity in the conjunctiva, cornea and tears [7]. Ocular surface inflammation results from complex interactions between innate (DCs, monocytes/macrophages, neutrophils, basophils, mast cells, eosinophils, natural killer cells, secretory IgA, members of the complement and clotting cascade, proteases and substrates) and adaptive (T and B cells, secretory immunoglobulins, mast cells) responses. Both immune responses show interactions and overlapping. For example, mucosal secretory IgAs protect the ocular surface by virtue of anti-adhesin properties displayed by

their heavy chains (innate response), through specific antigen-binding sites (adaptive response), as well as through augmented neutrophil-mediated amoebic lysis (adaptive immunity) [8].

Innate/adaptive cross-talk has recently been demonstrated to be driven by TLR expression on antigen-presenting cells and structural cells of the ocular surface. TLR expression has been found in the corneal and conjunctival epithelium, and this expression seems to be affected during bacterial/viral infections as well as in allergic conditions. The presence of TLRs in the ocular epithelium may be relevant for defence mechanisms towards microbial agents in contact with the ocular surface. It has been hypothesized that commensal flora, up till now regarded as a passive component of the external eye, may be critical for the maintenance of epithelial mucosal homeostasis, by playing a paradoxically protective role after epithelial injury, as suggested for the intestinal epithelium [9]. Moreover, changes in the commensal flora might influence the immune response in disease states. This hypothesis is supported by evidence that the gut flora of allergic children is different from that of non-allergic children. Certain microorganisms may thus actually be important for protection against allergy [10].

Toll-like receptors: history, structure, agonists and downstream signalling

Homologues of the *Drosophila* Toll genes, discovered in 1990, mammalian TLRs constitute a distinct and phylogenetically ancient class of the IL-1/TLR supergene family [11–13]. TLRs are transmembrane type I glycoproteins. They are characterized by an extracellular leucine-rich domain and a cytoplasm tail, homologous to the signal domain of the IL-1 receptor, which predominantly mediates the activation of mitogen-activated protein kinase and nuclear factor kappa B (NFκB)/activator protein 1 pathways and leads to cell activation and differentiation [4••,14,15]. A hallmark of the cell's response to the activation of innate immune systems is the release of TNF-α, IL-1β, and IFN-γ cytokines [4••,14].

Eleven mammalian and 10 human TLRs have been identified so far [14]. Most TLRs have been characterized according to their specific natural agonists, and, in general, TLR-2 and TLR-4 recognize bacterial products, whereas TLR-3, TLR-7, TLR-8 and TLR-9 are principally designed to join nucleic acids [5,16,17]. Regarding nucleic acid recognition, TLR-3 binds double-stranded RNA, typical of the replication process of some viruses [18•], TLR-7 and TLR-8 recognize single-stranded RNA, whereas TLR-9 binds unmethylated cytidine-phosphate-guanosine dinucleotide motives or synthetic analogues, rare in mammalian genomes but abundant in bacteria, fungi and viruses [19]. Specific natural or synthetic TLR agonists are listed in Table 1.

Table 1. A brief description of the main Toll-like receptor ligands characterized so far

TLR	Specific agonists
TLR-1	Triacylated lipopeptides
TLR-2	Gram-positive proteoglycan, zymosan, lipoproteins, mycobacteria and fungi
TLR-3	Double-stranded RNA (poly I : C) (viral)
TLR-4	Gram-negative lipopolysaccharide, taxol, respiratory syncytial virus F, HSP-60 (Chlamydia), fibronectin domain
TLR-5	Bacterial flagellin
TLR-6	Diacyl lipopeptides, zymosan
TLR-7	Single-stranded RNA, Imidazoquinoline (viral)
TLR-8	Single-stranded RNA, Imidazoquinoline (viral)
TLR-9	Microbial/viral unmethylated cytidine-phosphate-guanosine DNA
TLR-10	Not determined

HSP, Heat shock protein; TLR, Toll-like receptor.

TLRs can be classified as ubiquitous (TLR-1, TLR-6, TLR-7, TLR-8 and TLR-10), restricted (TLR-2, TLR-4 and TLR-5) and specific (TLR-3 and TLR-9). TLR-1 is expressed by all leukocytes [12]. TLR-2, TLR-4 and TLR-5 are largely expressed by myelo-monocytic cells [14]. TLR-3 is expressed in different tissues, but is restricted to DCs, monocytes/macrophages, intestinal epithelial cells and mast cells [14]. TLR-7, TLR-8 and TLR-9 are abundantly expressed by immune-competent or structural cells (epithelial cells and fibroblasts) populating the lung, placenta and spleen [20]. TLR-10 seems to be mainly expressed by lymphoid immune cells of the spleen, lymphnodes, thymus and tonsils [21]. Finally, mammalian TLR-11 (showing a Stop codon–open reading frame sequence) is highly expressed in the liver, bladder and kidney, but weakly expressed in the spleen (a pattern that differs from all other TLRs), and seems to be involved in responses towards uropathogenic *Escherichia coli*, responsible for infections of the urinary tract [22•].

Most of the TLRs recognizing bacterial products (TLR-2, TLR-4) reside on the cell surface [4••]. In addition to this common cell-surface localization, some TLRs favour intracellular localization, mainly at the endoplasmic reticulum and Golgi apparatus, implying active pathogen internalization and interchanging to tubular compartments [4••]. All nucleic acid-recognizing TLRs are expressed on endosomal membranes, rather than the outer membrane, and therefore this type of specific TLR binding occurs in the lumen of intracellular vesicles [4••]. For this reason, extracellular nucleic acids (from damaged tissues or infected/uninfected cells) must be endocytosed, whereas nucleic acids multiplying within a cell (bacterial/viral products) are captured in membranous vesicles, and then presented

to endosome-localized TLRs [4••]. Different TLR localization may also occur depending on cell type, reflecting the existence of multiple cell-specific pathways in antiviral recognition [18•]. For example, TLR-3 is intracellularly localized in immature DCs (multivescicular bodies and subcellular compartments of endocytic trafficking pathways), but cell-surface TLR-3 expression has also been observed in human fibroblasts, as an additional prompt way to recognize viruses [18•].

To begin the intracellular signalling cascade, TLRs require the presence of common or specific adaptor molecules (myeloid differentiation factor MyD88, TIRAP, TRAM, TRIF), recruited to the receptor complex to provide a platform for additional kinase-binding (IRAK-1/4, TRAF-6), which ultimately leads to mitogen-activated protein kinase activation and cytoplasmic NFκB translocation to the nucleus [4••,23–26,27••]. MyD88 represents the main adaptor molecule for most TLRs (TLR-2, TLR-4, TLR-7, TLR-8 and TLR-9) [24,28]. TLR-4-specific activation, throughout MyD88/NF-κB signalling, leads to the expression of pro-inflammatory cytokines (IFN, TNF-α, IL-1, IL-6, IL-12) and the upregulation of co-stimulatory molecules (CD40, CD80/B7-1 and CD86/B7-2) [29]. TLR-7, TLR-8 and TLR-9 specifically require MyD88, whereas some TLR-4 and TLR-3 pathways appear to be MyD88 independent [15,18•,23,25,30]. In particular, two other unexpected features of signalling have recently been described for TLR-3: Tyr-phosphorylation of the cytoplasmic domain of TLR-3 and the activation of phosphatidylinositol 3 kinase [18•]. Furthermore, SOCS1 (downregulating JAK/STAT/NFκB) and SOCS3 (induced by IL-6/IL-10/lipopolysaccharide stimulation) adaptor molecules, have recently been characterized [31].

Besides their individual contribution, some TLRs can also act as co-receptors (TLR-1, TLR-6, TLR-10) with other TLRs (TLR-2), and may promote or inhibit cellular responsiveness to specific agonists [31]. The co-transfection of different TLRs may lead to either enhancement or inhibition of ligand recognition, suggesting that cell responses to different agonists are dependent on the total repertoire of TLRs displayed on a cell, necessary co-factors, and levels of each protein present [31].

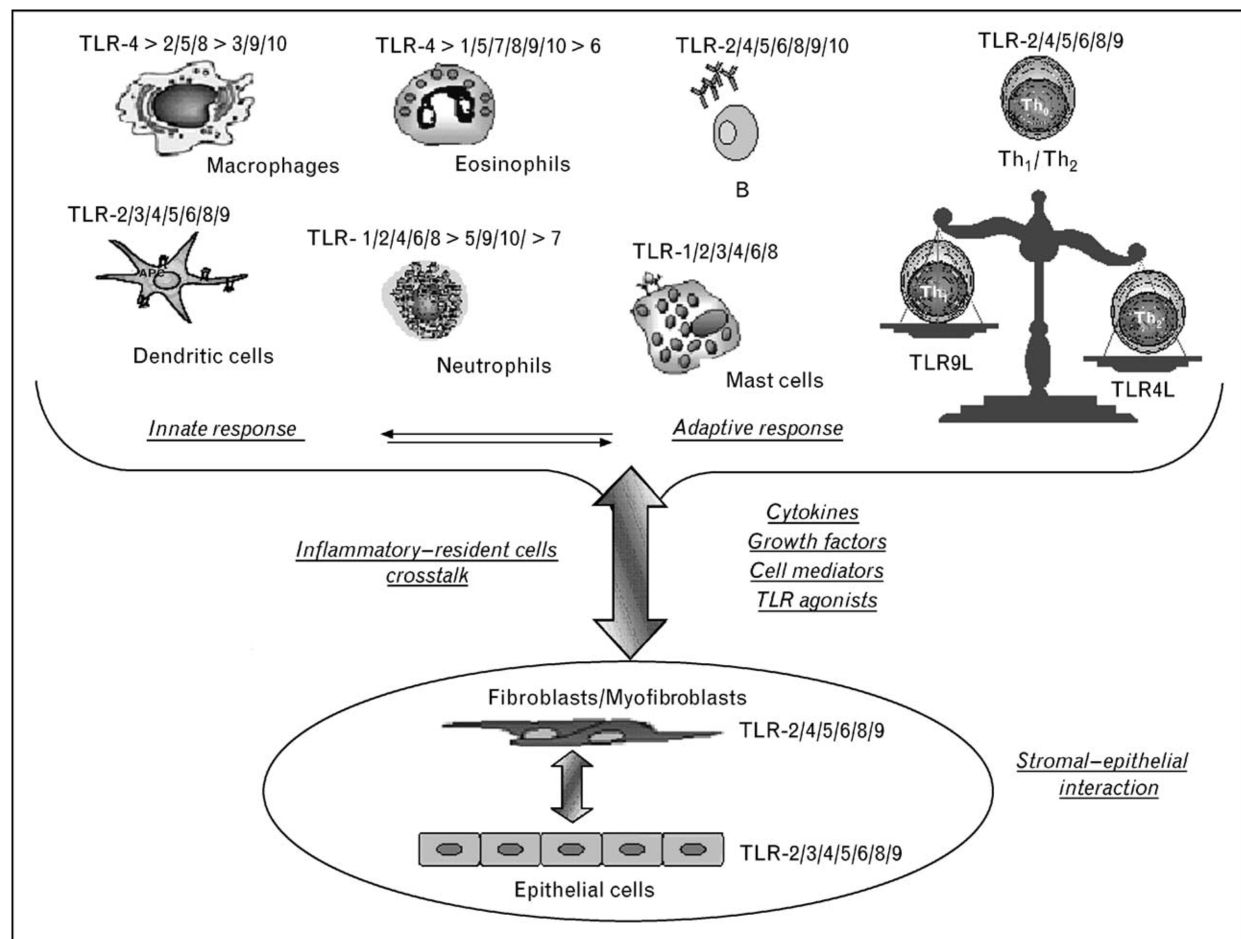
Toll-like receptors and immune cells: modulation of the immune response

Consistent with specific roles in innate immunity, TLRs are predominantly expressed in tissues involved in immune settings (tonsils, lymph nodes, spleen and thymus) as well as in tissues exposed to the external environment (skin, tracheobronchial epithelium, oral-gastrointestinal and urogenital tract, and corneal/conjunctival epithelium) [4••,32,33]. A difference in TLR expression may reflect different functional tasks in the local immune response [1]. At the cellular level, TLRs have been found to be widely expressed by structural cells (epithelial cells, fibroblasts and endothelial cells) and immune cells (antigen presenting/DCs, monocytes/macrophages, T and B lymphocytes, neutrophils, eosinophils and immature/mature mast cells). A schematic representation of TLR expression by structural and inflammatory cells, populating the eye under physiological and pathological conditions, is reported in Figure 1.

The pattern of TLR expression in different peripheral blood leukocytes implies specific roles in each population. Mononuclear phagocytes express particularly high levels of TLR-4, as well as TLR-2, TLR-3, TLR-7, and TLR-9 [34]. Macrophages/monocytes, and to a lesser extent, granulocytes express higher levels of TLR-2, TLR-4, TLR-5, and TLR-8, and lower levels of TLR-3, TLR-9 and TLR-10 compared with B cells, whereas their TLR-1, TLR-6, and TLR-7 expression seems to be almost comparable [35]. B cells express the highest levels of TLR-9 and TLR-10 compared with other blood cells [36]. Neutrophils, which represent the first cells involved in immune responses, express high levels of TLR-1, TLR-2, TLR-4, TLR-6, TLR-8, average levels of TLR-5, TLR-9, TLR-10, and low levels of TLR-7 [37–39].

After TLR binding to pathogens, DCs, specializing as antigen-presenting cells, migrate from the periphery to lymphoid structures, upregulating MHC and co-stimulatory molecules, and priming pathogen-specific naive lymphocytes [40]. TLR-dependent DC activation and antigen processing/presentation represents a necessary step for the development of adaptive T helper type 1/2 and B responses [41]. DC activation is followed by the release of different cytokines, able to drive the polarization of T cells into T helper type 1, 2 or regulatory phenotypes, a critical task for defence against pathogens [42]. Adaptive responses require a well-balanced bacterial/viral (T helper type 1) as well as parasite/helminth (T helper type 2) recognition. Any deregulation/impairment in this balance may drive autoimmune (T helper 1) or allergic (T helper 2) disorders. The observation that TLR-4 seems to be necessary to develop an optimal T helper type 2 response, that TLR-9 strongly induces a T helper type 1-biased response, and finally that TLR-2 can drive T helper type 2-biased adaptive immune responses, exacerbating the allergic phenotype suggests that different TLRs may induce opposite T helper type 1/2 polarizations [29,43,44]. Moreover, DCs expressing TLR-3, TLR-5 and TLR-7 directly assist the activation/differentiation of naive CD4 T cells into either T helper type 1 or 2 effector/memory cells capable of producing either IFN-γ or IL-4, IL-5, and IL-13 [43]. These findings have generated a growing interest in

Figure 1. Structural and inflammatory cells and their specific Toll-like receptors



All structural and inflammatory cells, taking active part in both innate and adaptive immune responses, are displayed in relation to specific Toll-like receptor (TLR) expression. In particular, the possible mechanisms underlying T helper type 1/2 biased responses, are highlighted.

defining how the pathogen/TLR relationship on DCs may regulate T helper type 1 versus type 2-mediated adaptive immunity [43].

In addition, TLR pathways may mediate interactions between DCs, T lymphocytes and mast cells/eosinophils, thus modulating allergic responses [43]. The role of lipopolysaccharide exposure in the induction/modulation of existing asthma has been described but is poorly understood, and seems to be highly complex and dependent on several parameters. Lipopolysaccharide treatment in experimentally induced asthma prevents the onset of T helper type 2 responses and pulmonary inflammation as well as the associated airway hyperreactivity, but on the other hand, if given before antigenic sensitization, it may increase IgE production [43]. The possible inhibitory effects of lipopolysaccharide on the T helper type 2 response are consistent with lipopolysaccharide inducing IL-12 and IFN-γ, which in some allergen-induced asthma models block T helper type 2, and favour T helper type 1 responses [43,45].

Eosinophils, constantly associated with a T helper type 2 phenotype, constitutively express high levels of TLR-4, average levels of TLR-1, TLR-7, TLR-8, TLR-9, TLR-10 and low levels of TLR-6 [46]. The TLR-7/8 pathway regulates adhesion molecules (CD11b, L-selectin), and survival and superoxide generation by eosinophils, albeit their natural ligands have not yet been identified [46]. Interestingly, eosinophils represent the first evidence that a bi-directional link exists between TLRs and cytokines: TLRs are able to modulate cytokine production, and TLRs can be in turn modulated by the cytokines present in the micro-environment [43,45]. This aspect was highlighted by the increase in TLR-7 expression caused by IL-6, and the increase in TLR-8 expression caused by IFN-γ, in cultured eosinophils [46].

Human mast cells, key cells exerting a crucial role in host defence against pathogens and in allergic responses, express TLR-1, TLR-2, TLR-4, TLR-6, TLR-8, but not TLR-5 [47]. Specific TLR-2 and TLR-4 activation

induces mast cell degranulation and T helper type 2 cytokine release [48]. In cord blood-derived mast cells, TLR-2 activation is followed by the release of granulocyte macrophage colony-stimulating factor, IL-1β and leukotrienes, whereas TLR-4 activation does not induce a degranulating response [47]. Interestingly, human cultured mast cells also express TLR-3, and, upon stimulation, induce type I IFN-γ release, indicating a potential mast cell contribution to the innate immune response to viral infections [49,50]. In addition, it has recently been reported that platelets express TLR-2, TLR-4 and TLR-9, representing a further link between innate and adaptive TLR responses [51].

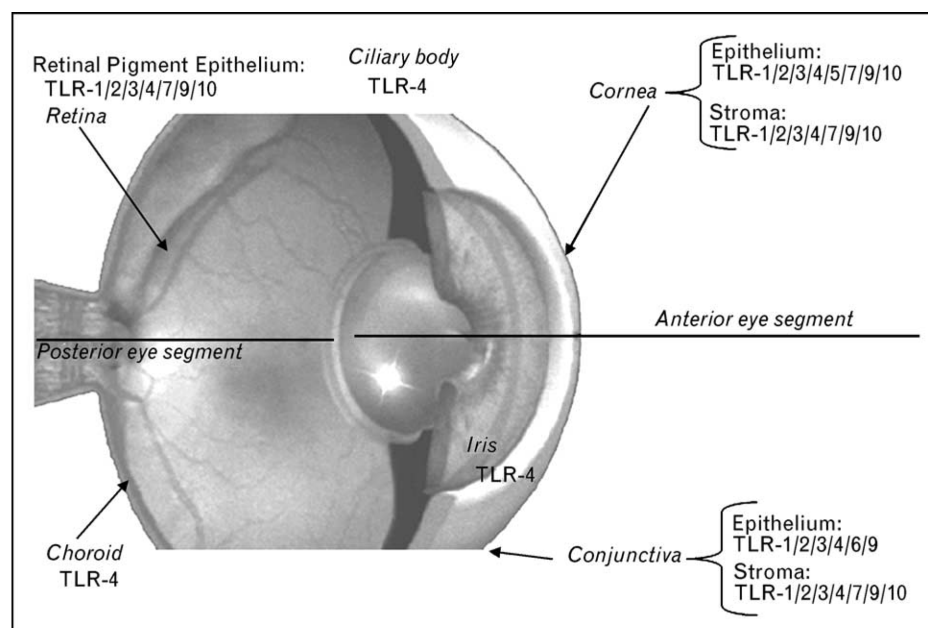
Toll-like receptors and the eye: across infection, towards allergy

The widespread TLR expression in the eye is outlined in Figure 2. Human retinal pigment epithelial (RPE) cells express high levels of TLR-2, TLR-3 and TLR-4, co-stimulatory CD14 and MD2 molecules, as well as TLR-1, TLR-7, TLR-9 and TLR-10 [52••]. According to the localization, RPE cells play different roles, including structural (basement of the retina) and functional (nutrient transport) activities under physiological conditions, as well as in a variety of pathological processes of the retina, associated with the early damage of epithelial cells [53,54]. RPE cells take an active part in the immune response of the retina, as a first line as well as an adaptive defence. They may function as antigen-presenting cells, and upon stimulation, represent a good source of chemokines, cytokines and growth factors that can exert opposite effects during pathological processes (for example during cytomegalovirus, *Toxoplasma gondii*, and coronavirus infections) [55]. It has been observed that TLR-2, TLR-3 and TLR-4 expression by RPE cells is upregulated by TLR-3 agonists or IFN-γ [52••]. In particular, TLR-3 agonists induce the release of IFN-β (highly inhibiting viral replication), IL-6, IL-8, monocyte chemoattractant protein 1, and soluble intercellular cell adhesion molecule 1. This evidence is in line with the current findings regarding TLR-3 restricted distribution and functional activity.

Interestingly, TLR-4/CD14/MD2 expression, associated with lipopolysaccharide receptor complex, has been found in resident antigen-presenting cells populating the human choroid, ciliary body, iris and sclera [56].

Physical or chemical injury can compromise ocular surface integrity and allow the entry of live organisms and microbial products to the underlying epithelium and to the stroma, where they can induce an inflammatory response that might lead, if unbalanced or chronic, to damage, visual impairment and possibly blindness [57,58]. The corneal and conjunctival epithelia represent a critical barrier of the ocular surface to the outer environment, protecting the eye from pathogens. Corneal epithelial cells express TLR-4 and co-stimulatory molecules (CD14, MD2), and stimulation by TLR-4 agonists results in pro-inflammatory cytokine and chemokine secretion [44]. Interestingly, the corneal epithelium does not normally respond to commensal flora, although commonly

Figure 2. Diagram outlining Toll-like receptors distribution and signalling in the eye



TLR, Toll-like receptor.

present in the tear film, as observed by the fact that patients suffering from bacterial conjunctivitis do not display corneal inflammation. The corneal epithelium appears to posses a unique way (intracellular localization) to modulate the functional activity of the highly expressed TLR-2 and TLR-4, and therefore to control unnecessary inflammation [59]. In fact, corneal epithelial cells do not express TLR-2 and TLR-4 at the cell surface, failing to elicit immune response to ligands (TLR silent form) [59]. TLR agonists may, however, lead to corneal keratitis, as a result of specific neutrophil recruitment and the onset of an unbalanced local inflammatory response [41]. A TLR-5 agonist, *Pseudomonas aeruginosa* (an opportunistic Gram-negative pathogen), is able to cause bacterial keratitis in contact-lens wearers [60]. Interestingly, corneal TLR-4 activation might have both beneficial and detrimental effects, depending on the magnitude/duration of stimulation and the effectiveness in the removal of the pathogen: the fragile balance between these two opposite actions is responsible for eventual corneal scarring and blindness [44].

Corneal ulcer, caused by bacterial infection, represents the major cause of vision loss, as a result of the destruction of collagen fibrils in the corneal stroma by collagenolytic enzymes [44]. Infiltrated leukocytes (neutrophils and macrophages), surrounding or penetrating the corneal ulcer, may contribute to collagen degradation by interacting with activated keratocytes [44]. In a recent study, the role played by TLR-4/CD14/MD-2-expressing fibroblasts (activated keratocytes) in lipopolysaccharide-induced inflammation associated with bacterial corneal ulceration, has been demonstrated [61]. Chemokines, IL-8 and monocyte chemoattractant protein 1, as well as intercellular cell adhesion molecule 1, are all actively produced by activated keratocytes [61]. Such studies imply that specific lipopolysaccharide recognition by keratocytes and their consequent activation contribute to bacterial corneal ulceration [61]. By using gene-manipulated mice ($TLR2^{-/-}$, $TLR9^{-/-}$, $MyD88^{-/-}$), it was observed that during the development of corneal inflammation, epithelial cells express functional TLR-4, TLR-2 and TLR-9, and their specific activation in keratitis was entirely MyD88 dependent [27••].

In agreement with its role as a first line of defence, the healthy conjunctival epithelium expresses high levels of TLR-9, compared with the average expression of TLR-2 and TLR-4, whereas the expression by the underside stroma is at similar levels [62••]. This expression is modified in patients with vernal keratoconjunctivitis (VKC), a chronic allergic inflammation of the ocular surface affecting pre-pubescent children, characterized by the activation of cytokines and inflammatory mediators (infiltration of mast cells, eosinophils and lymphocytes in the conjunctival epithelium and stroma) [62••]. Real-time evaluation of VKC conjunctiva showed a significant upregulation of TLR-4 and downregulation of TLR-9, with a slight reduction in TLR-2, compared with healthy conjunctiva [62••]. Confocal analysis showed that in VKC, stromal TLR-4 expression was mainly caused by fibroblasts, infiltrating eosinophils and mast cells. High levels of TLR-4 expression in VKC tissues is substantiated by previous reports correlating TLR-4 expression to the allergic phenotype. The noteworthy downregulation of TLR-9 at the molecular level was supported by the massive disappearance of TLR-9 staining in the epithelium of VKC biopsies, compared with healthy samples [62••]. However, significant gaps still remain in understanding the mechanisms underlying the downregulation of TLR-9 in VKC epithelium.

Consistent attention has therefore been devoted to the potential use of immunostimulatory sequence oligodeoxynucleotides (ISS-ODN) in the treatment of allergic diseases [54]. Experimentally, TLR-9 agonists (cytidine-phosphate-guanosine, or cytidine-phosphate-guanosine dinucleotide motives or synthetic analogues DNA) exert immune stimulatory activity, biasing the immune response towards the development of an antigen-specific T helper type 1 response [63]. As conjunctival allergy results from a prevalent T helper type 2 response to allergens, and in accordance with the hypothesis that skewing the T helper type 1/2 response to allergens in patients suffering from seasonal allergic conjunctivitis or VKC could have significant therapeutic value, it has recently been reported that the systemic or conjunctival administration of ISS-ODN in allergic mice significantly inhibited acute allergic conjunctivitis and completely inhibited both neutrophils and eosinophils in the late-phase reaction, suggesting that ISS-ODN may be an effective therapeutic agent for this set of allergic diseases [54,64].

Conclusion

The novel and rapidly growing field of TLR investigation may ultimately lead to a better understanding of the molecular basis linking infection and immunology. Besides describing several data present in the literature regarding the characterization, structure and signalling pathways of TLRs, highlighting the crucial role of TLRs in innate and adaptive response, this review discusses the first description of TLRs in the human ocular surface, during physiological and pathological conditions. Recently, the hypothesis that at least in the development of asthma, early life specific activation of TLRs may contribute to a more balanced T helper type 1/2 response, avoiding overactivation of the T helper type 2 pathway, has been proposed. In line with this hypothesis, further studies of the role of commensal flora in influencing innate immunity in the eye, mainly during the early stages, may lead to a re-evaluation of the mechanisms

that activate the adaptive immune responses after microbial ocular infections. Finally, pharmacological activation and regulation of TLRs may offer new therapeutic alternatives for the modulation of allergic and immune responses.

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Nerve growth factor effect on human primary fibroblastic-keratocytes: Possible mechanism during corneal healing

Alessandra Micera [a,b], Alessandro Lambiase [a], Ilaria Puxeddu [b], Luigi Aloe [c], Barbara Stampachiacchiere [a], Francesca Levi-Schaffer [b], Sergio Bonini [c,d], Stefano Bonini [a,*]

[a] *CIR Laboratory of Ophthalmology, University Campus Bio-Medico and G.B.Bietti, Foundation, Via Emilio Longoni 83, 00155 Rome, Italy*
[b] *Department of Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel*
[c] *Institute of Neurobiology and Molecular Medicine, National Research Council, Rome, Italy*
[d] *IRCCS San Raffaele, Rome, Italy*

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Abstract

In response to corneal injury, cytokines and growth factors play a crucial role by influencing epithelial-stromal interaction during the healing and reparative processes which may resolve in tissue remodeling and fibrosis. While transforming growth factor-β1 (TGF-β1) is considered the main profibrogenic modulator of these process, recently the nerve growth factor (NGF) appears as a pleiotropic modulator of wound-healing and inflammatory responses. Interestingly in the cornea, where NGF, $trkA^{NGFR}$ and $p75^{NTR}$ are expressed by epithelial cells and keratocytes, the NGF eye-drop induces the healing of neurotrophic or autoimmune corneal ulcers. During corneal healing, quiescent keratocytes are replaced by active fibroblast-like keratocytes/myofibroblasts. While the NGF effect on epithelial cells has been investigated, no data are reported for NGF effects on fibroblastic-keratocytes, during corneal healing. NGF, $trkA^{NGFR}$ and $p75^{NTR}$ were found expressed by fibroblastic-keratocytes. NGF was able to induce fibroblastic-keratocyte differentiation into myofibroblasts, migration, Metalloproteinase-9 expression/activity and contraction of a 3D collagen gel, without affecting their proliferation and collagen production. These data also show a two-directional control of fibroblastic-keratocytes by NGF and TGF-β1. To sum up, the findings of this study indicate that NGF can modulate some functional activities of fibroblastic-keratocytes, thus substantiating the healing effects of NGF on corneal wound-healing.
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Keywords: corneal healing; fibroblastic-keratocyte; NGF; tissue repair; metalloproteinases

1. Introduction

After corneal injury, epithelial cells, surrounding the injured area, proliferate and migrate to replace the damaged area (Wilson et al., 2001; Schultz et al., 1994). All at once in the stroma, quiescent keratocytes become activated inside and around of the wounded area, by their conversion into motile and highly active producers of collagenases, proteases, extra cellular matrix components, allowing the reconstruction of damaged stroma (fibroblastic-keratocytes), and later on into myofibroblasts (myoFBs, identified by their αSMA expression), showing the ability to promote wound closure (Wilson et al., 1999, 2001: Jester and Ho-Chang, 2003; Imanishi et al., 2000). These phenotypical conversions/differentiations occur in view of the changed microenvironment, due to the presence of different cytokines and growth factors (Wilson et al., 1999, 2001; Visse and Nagase, 2003; Jester and Ho-Chang, 2003), and their related receptors, which play a crucial role in wound-healing processes (Wilson et al., 1999). The healing process comprises also the production/deposition of

Abbreviations: NGF, nerve growth factor; TGF-β1, transforming growth factor β1; myoFBs, myofibroblasts; ECM, extra-cellular matrix; MMPs, metalloproteinases; PBS, phosphate-buffered saline.

* Corresponding author. Tel.: +39 6 2254 1342; fax: +39 6 2254 1456.
E-mail address: s.bonini@unicampus.it (S. Bonini).

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Extra-cellular Matrix (ECM) components, as well as degradation and clearance of newly synthesized products by Metalloproteinases (MMPs; Wilson et al., 2001; Ye and Azar, 1998). Transforming growth factor β1 (TGF-β1) represent one of the most important modulator of corneal wound-healing, being able to induce proliferation, differentiation/survival, collagen production by FBs/myoFBs (Frank et al., 1996; Ling and Robinson, 2002). Old and recent studies have shown that also the nerve growth factor (NGF), the most well studied member of a neurotrophin family, plays a crucial role in modulating wound-healing process in cornea and skin (Lambiase et al., 2004; Micera et al., 2004). In the ocular surface, NGF, $trkA^{NGFR}$ and $p75^{NTR}$ have been observed in both healthy cornea and conjunctiva (You et al., 2000; Lambiase et al., 1998a). The specific NGF binding to $trkA^{NGFR}$ and $p75^{NTR}$ elicits complex signal transduction pathways ranging from proliferation/differentiation to survival/apoptosis (Micera et al., 2003). Interestingly, the treatment with NGF eye-drop stimulates corneal healing in patients affected by neurotrophic or autoimmune corneal ulcers (Lambiase et al., 1998b, 2000a; Bonini et al., 2000;). Even though, NGF healing activity of corneal ulcers has been described at the clinical level, scarce data exists on the mechanisms leading to corneal recovery at the cellular level, and most of them are related to proliferative/differentiative effects of NGF on corneal epithelial cells (You et al., 2000; Lambiase et al., 2000a). Since in wounded cornea quiescent keratocytes, give rise to specialized fibroblastic-keratocytes and myoFBs, to attempt the healing process (Wilson et al., 2001), the aim of this study was to investigate in vitro the effect of NGF on primary fibroblastic-keratocytes, by evaluating cell differentiation, migration, proliferation and functional activity (ECM metabolism) in both mono and 3D conditions.

2. Materials and methods

2.1. Primary cell culture

According to several in vitro studies: i) primary quiescent keratocytes grown in the absence of serum; ii) the active keratocytes (also named fibroblastic-keratocytes) are obtained from keratocytes in the presence of 10% serum (less then 10–20% differentiation into myoFBs); and iii) finally myoFBs are obtained after stimulation with 0.25–1 ng/mL TGF-β1 (Jester et al., 1999; Jester and Ho-Chang, 2003). For all the studies reported herein, cultured keratocytes are referred as active or fibroblastic-keratocytes.

Primary fibroblastic-keratocytes were isolated from cadaver corneal biopsies, following the explants procedure (Veneto Eye Bank, Italy, $n = 9$). Briefly, the epithelial and endothelial layers were mechanically removed and the stroma were placed as explants in 24 wells at 37 °C, 5% CO_2 in air, in the presence of 10% heat-inactivated fetal bovine serum (FBS) medium (200 μL medium: DMEM, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin), according to a previous protocol to give rise to fibroblastic-keratocyte (Beales et al., 1999; Jester and Ho-Chang, 2003; Hao et al., 2005; Armstrong et al., 2003). Growing cells were trypsinized (0.2% trypsin/0.025% EDTA), sub-cultured and used for experiments up to seven passages. The purity was assessed by the anti-keratin 3 epithelial marker exclusion test (AE5, 1/150, ICN, Germany). All sterile tissue culture plastic-ware and reagents were from Iwaki (Iwaki Co., Tokyo, Japan), NUNC (Roskilde, Denmark), SIAL (Rome, Italy), Celbio (Milano, Italy) and Invitrogen-GIBCO (Carlsbad, CA, USA), otherwise specified in the text.

2.2. Stimuli and experimental procedure

For the experiments, all the cells were replated at high density conditions ($1 \times 10^5/cm^2$). Serum starved monolayers (0.5% FBS-medium for 24 h) were incubated for 2 to 6 days (replaced every two days) with or without the following additives: NGF (murine mouse β-NGF; 5, 10, 25, 50, 100, 250, 500 ng/mL; prepared/purified according to a standard procedure (Bocchini and Angeletti, 1969); neutralizing mouse anti-NGF antibodies (AbNGF 1 μg/mL; R&D Systems, Minneapolis, MN, USA); human TGF-β1 and neutralizing anti-panTGF-β (TGF-β1: 1, 5, 10, 15, 20 ng/mL; and AbTGFβ: 0.5, 1, 10 and 25 μg/mL; both from R&D); or pre-incubated with specific neutralizing antibodies (AbNGF and AbTGFβ, both 1 μg/mL or 0.5 μg/mL) for 1 h at 37 °C before the addition of the specific growth factors, either alone or combined, for 2 to 6 days.

2.3. Immunostaining analysis

Round coverslips (Mierfield, USA) confluent monolayers were exposed to NGF, TGF-β1 or medium alone, for 20 h, 2 or 6 days. Monolayers were washed in Hank's Balanced Sodium Salt (HBSS), fixed in 2% ρ-formaldehyde (PFA) diluted in 10 mM phosphate-buffered saline (PBS), briefly permeabilized in 0.5% Triton X-100 diluted in PBS (TX-PBS) and processed for immunofluorescence. The following specific antibodies, diluted in PBS, were used: rabbit anti-human $trkA^{NGFR}$ antibodies (1/100; Santa Cruz Biotech., CA, USA); goat anti-human $p75^{NTR}$ antibodies (1/100; Santa Cruz) and monoclonal anti-human αSMA antibodies (1/50; Novocastra, Milan, Italy). Specific binding of the primary antibody was detected using secondary biotinylated antibodies, and labeled with FITC-Avidin D (2 μg/mL; Vector Laboratories, Inc., Burlingame, CA, USA), by means of the Avidin Biotin Complex technique (Vectastain Elite II ABC kit, Vector Laboratories). In some experiments, phycoerythrin (PE)-labeled anti-mouse secondary antibody (20 μg/mL, Vector Laboratories) was used for α-SMA primary antibody. The nuclei were visualized by Hoechst 332581 (1 μM; Sigma-Aldrich, Rome, Italy). Coverslips were mounted using an anti-fade solution (AF1, Cityfluor, Cambridge, UK). Control isotype was performed by substituting primary antibody with control irrelevant rabbit/goat/mouse IgG (Vector Laboratories). Fluorescence images were collected with a E2000U confocal microscope (Nikon, Tokyo, Japan). Acquisition and brightness/contrast levels were carried out using respectively

the C1 software (Nikon) and the Adobe Photoshop 7.0 program (Adobe Systems Inc., San Jose, CA, USA).

2.4. RNA isolation and conventional RT-PCR

Total cell RNA was extracted from confluent not-pooled monolayers (3×10^6 cells) using the TRIzol technique (Invitrogen-GIBCO), re-suspended in 25 μL diethyl pyrocarbonate-treated water (Sigma-Aldrich, St. Louis, MO) and treated with RNase-Free DNase I (2 U/μL Turbo DNA free kit AM-1907; Ambion Ltd., Cambridgeshire, UK), to remove DNA contamination. Total RNAs were spectrophotometer evaluated ($\lambda_{260}/\lambda_{280} > 1.8$), 1% agarose-ethidium bromide size-fractionated and photographed by a Kodak imager station (Kodak 550, Eastman Kodak Company, Sci. Imaging Systems, Rochester, NY) to confirm absence of RNA degradation. Normalized RNAs were used as a template in cDNA synthesis. Following reverse transcription of 3 μg total RNA by the SuperscriptII kit (final volume reaction of 20 μL, using 50 pM oligo dT_{21}-primer, 1 mM dNTP mix and 200 U reverse transcriptase; GIBCO-Invitrogen), the resulting cDNA was amplified using Hot-start Pfx Platinium DNA polymerase and a dNTP mixture (all from GIBCO-Invitrogen), in a PT-100 programmable thermocycler (MJ Research, Watertown, MA). Specific primers for human NGF, $trkA^{NGFR}$, $p75^{NTR}$ and GAPDH mRNAs were designed using Primer3 software (available at the http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), possibly to be one intron-spanning. GenBank software was used to select the complete mRNA sequence of each gene investigated http://www.ncbi.nlm.nih.gov/Genbank; provided by the National Center for Biotechnology Information, Bethesda, MD). Primer sequence (MWG Biotech, Ebersberg, Germany), PCR product size, amplifying conditions and GenBank Access Number for each primer, are listed in Table 1 (conventional PCR section). Since the housekeeping gene GAPDH was found to be constantly expressed in our experiments, all the samples were amplified with GAPDH primers and all PCR products were normalized to GAPDH amount (referring gene). Negative controls (without template) were produced for each run. PCR products were size-fractionated in a 2.5% agarose gel and photographed in a Kodak imager station (Kodak 550). Images were captured digitally using the 1D Kodak Image Analysis Software, subject to densitometric analysis and processed in Adobe Photoshop 7.0.

2.5. Relative real-time PCR

Real-time PCR experiments utilized total RNA obtained according to Trizol extraction protocol. In the case of collagen gels, the gels were pretreated with collagenase VII to release the cells from the scaffold (500 μL of collagen buffer, 2 U/mL at 37 °C for 60 min; Berton et al., 2000). Total RNA (3 μg; purity λ260/λ280 ≥1.8) was used as template for cDNA synthesis as reported for conventional PCR. The SYBR Green PCR core reagents kit (Applied Biosystems, Foster City, CA) was used for the PCR reaction, carried out using Opticon2 MJ Research system (MJ Research). The reaction contained 10 μL of SYBR reagent, 3 μL of cDNA (for the target) or 1 μl of cDNA (for the referring gene), and 20 nM primers in a 20-μL final volume. αSMA and MMP-9 primer specifications and the thermal cycling conditions are listed in Table 1 (real-time PCR section). Experiments were performed in triplicate for each data point. Quantitative values were obtained from the threshold cycle value (Ct), which is the point where a significant increase of fluorescence is first detected. The transcript number of GAPDH was quantified as an internal RNA control, and each sample was normalized on the basis of its GAPDH content. According to the REST©

Table 1
Sum up of the primers and amplifying conditions used in the conventional and real-time PCR assays

Primer	Sequence	PCR product (size bps)	Annealing conditions*	Genbank Access no.
For conventional PCR				
NGF	for: 5′-CTG GCC ACA CTG AGG TGC AT-3′	120 bps	53 °C, 30 s	V01511
	rev: 5′-TCC TGC AGG GAC ATT GCT CTC-3′			
$trkA^{NGFR}$	for: 5′-TGG CTG ATA CTG GCA TCT GCG-3′	277 bps	55 °C, 25 s	M23102
	rev: 5′-AGC CGA GGA GTG AAA TGG AAG G-3′			
$p75^{NTR}$	for: 5′-GAG GCA CCA CCG ACA ACC TC-3′	120 bps	55 °C, 25 s	AF187064
	rev: 5′-TGC TTG CAG CTG TTC CAC CT-3′			
GAPDH	for: 5′-GAA GGG GTC ATT GAT GGC AAC-3′	100 bps	53 °C, 30 s	BC013310
	rev: 5′-GGG AAG GTG AAG GTC GGA GTC-3′			
For real-time PCR				
TGF-β1	for: 5′-TCC TGG CGA TAC CTC AGC AA-3′	110 bps	53 °C, 30 s	BC017288
	rev: 5′-GCC CTC AAT TTC CCC TCC AC-3′			
α-SMA	for: 5′-GAA GGA GAT CAC GGC CCT A-3′	125 bps	60 °C, 25 s	BC017554
	rev: 5′-ACA TCT GCT GGA AGG TGG AC-3′			
MMP-9	For: 5′-GCT CAG TCG CCT GAA TCT CT-3′	113 bps	60 °C, 25 s	BC006093
	Rev: 5′-GCA CAA GAA CAG TGC AGA GG-3′			

The identity of amplified fragments was assessed by Southern Blotting, according to a standard procedure. Hot-start Pfx Platinium DNA polymerase and AmpliTaq Gold were activated by an incubation for 10 min at 95 °C. Each of the 35–55 PCR cycles consisted of a 30 s of denaturation at 95 °C, followed by a specific annealing step* and a 30 s of extension at 72 °C. Melting reading for real-time PCR was set as follows: reading from 56.0 C to 94.1 C; 0.3 C; hold for 00:00:01 between reads.

software (Pfaffl et al., 2002), results are expressed as N-fold difference (increase or decrease) in target gene expression, normalized to referring gene expression, compared to controls. The specificity of each PCR products were confirmed by the single melting curves obtained during amplification.

2.6. Protein analysis: ELISA, Western blotting and SDS-PAGE zymography

To evaluate NGF in the culture media, a two site NGF-ELISA, with a sensitivity of 0.5 pg/mL, was performed following the Weskamp and Otten (1987), with minor modifications. In brief, ELISA plates (Maxisorp NUNC 96 well plates) were coated with mouse anti-NGF antibodies (0.4 μg/mL; MAB5260, R&D). After binding treatments, the standards ranged from 0.15 pg/mL to 1 ng/mL NGF, polyclonal anti-NGF antibodies (0.15 μg/mL, PeproTech EC Ltd, London, UK) and streptavidin solution (1/10,000; Zymed, San Francisco, CA, USA). After the addition of 3,3′,5,5-TetraMethylBenzidine (TMB, Zymed), the optical density (at 450 corrected for 550) was detected by an ELISA reader (Sunrise, Tecan Systems, Inc., San Jose, CA, USA). Under these conditions, no cross reactivity with Brain Derived Neurotrophic Factor (BDNF) or Neurotrophins 3/4/5 was observed. In addition, the biological activity of NGF released in the conditioned media by fibroblastic-keratocytes, was tested separately by using a PC12 bioassay (Green, 1977), whose specificity was confirmed by adding neutralizing monoclonal anti-NGF antibodies (1 μg/mL, R&D) to the replicate samples. The levels of TGF-β1 in the media were measured using a commercial ELISA kit, according to the manufacturer' procedures (Biosource International, Camarillo, CA, USA). Normalization was achieved by the DC protein assay kit (Bio-Rad Laboratories, Inc, Hercules, CA, USA).

Western blot analysis for $trkA^{NGFR}$ (1/700), $p75^{NTR}$ (1/500), collagens I and IV (1/1000; anti-goat antibodies from Santa Cruz) and αSMA (1/500) were carried out on protein extracted in 1 mL of ice-cold lysis buffer (25 mM Tris–HCL pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 10 mM NaF, 10 μg/mL Aprotinin, and 1 mM PMSF). 30 μg protein were subjected to SDS-PAGE electrophoresis (Miniprotean3 apparatus, Bio-Rad) and resolved proteins were transferred to Hy-bond membranes in a semi-dry blotting apparatus (Bio-Rad). Membranes were pretreated with PBS containing 5% non-fat dry milk and washed in 0.05% Tween 20 in PBS (TW-PBS). Transferred proteins were probed with primary antibodies diluted in TW-PBS, followed with secondary HRP-conjugated antibodies (1/8000, Zymed) and ECL developed (SuperSignal West Pico Trial, Pierce) in a Kodak imager station (Kodak 550). Images were captured digitally using the 1D Kodak Image Analysis Software, subject to densitometric analysis and processed by Adobe Photoshop 7.0.

MMP activity in the conditioned media was analyzed by zymography, as described previously (Berton et al., 2000). Briefly, conditioned media were collected, clarified by centrifugation and mixed with SDS sample buffer without β-mercaptoethanol and incubated 12 h at 4 °C. Normalized samples were heated for 30 min at 37 °C and electrophoresed in a 10% SDS-PAGE containing 0.1% gelatin (Bio-Rad). Recombinant human latent and active MMP-9 were loaded as positive controls (Berton et al., 2000). The gels were washed in 2.5% TX-PBS to remove SDS, incubated for 24 h at 37 °C in 200 mM NaCl containing 40 mM TB pH 7.5 and 10 mM $CaCl_2$. The gels were fixed for 1 h (40% MeOH, 10% Acetic Acid, 50% deionized water), stained for 2 h in Coomassie Colloidal (ICN), enhanced for 10 min in 8% acetic acid and 25% EtOH in deionized water, digitally acquired and finally subject to densitometric analysis. The presence of gelatinolytic activity was identified as clear bands on an uniform blue background following destaining in 10% Acetic Acid solution for 2 h.

2.7. Cell surface ELISA

To investigate the effect of NGF on αSMA expression, a cell surface ELISA test was carried out (Micera et al., 2001). Briefly, the cells (5×10^5/0.20 mL) were seeded in NUNC 96 well culture-treated plates, grown until confluent, and then incubated with different additives. After 20 h, 2, 4 or 6 days of culture, the plates were washed in HBSS containing 0.1% $CaCl_2$, post-fixed in 2% buffered PFA, quenched for endogenous peroxidases and dehydrated with 95% ethanol before the assay. The plates were subsequently processed for quantification of αSMA (1/500), collagens I and IV (1/1000), using monoclonal and polyclonal specific antibodies diluted in TW-PBS. Biotinylated anti-mouse or anti-goat antibodies and HRP-streptavidin (1/8000 and 1/20,000, Zymed) specific binding were developed with a ready to use TMB solution as substrate (Zymed) and quantified using Sunrise ELISA reader.

2.8. Proliferation assays

The effect of NGF on cell proliferation was first investigated by counting cells after brief enzymatic digestion (trypan blue exclusion test). As an independent index of proliferation, 2% buffered PFA fixed monolayers were immunostained for ki67, using a rabbit anti-human ki67 antibody (1:1000, Santa Cruz), according to the ABC technique. Ki67 is a nuclear proliferation factor expressed at all the cell cycles except G_0 (Joyce et al., 1996). More specifically, $[^3H]$-thymidine uptake was also investigated (Micera et al., 2001). In brief, subconfluent (4×10^3/0.2 mL) and confluent (1×10^4/0.2 mL) monolayers were incubated with media containing $[^3H]$-thymidine 1 μCi/mL, without (control) or NGF (0–500 ng/mL) or TGF-β1 (10 and 50 ng/mL) for 1 day. Cells were then lysed, washed, 5% TCA precipitated, Et-OH washed, dissolved in 1N NaOH and finally, after the addition of liquid scintillation, radioactivity was counted in a beta-counter (JKB 1211 Rackbeta).

2.9. Migration assay

Migration of wounded fibroblastic-keratocytes was assessed using an in vitro model in which a linear wound midline

was produced in a confluent monolayer using a razor blade and half of the monolayer was then scraped free of cells (Levi-Schaffer and Kupietzky, 1990). After wounding and removal of supernatants, different concentrations of additives alone or in combination were added with fresh medium. Cell migration across the wound line was then estimated after 2, 4, 12 and 24 h, by counting the number of optic grids from the wounded line to the farthest migrating cells under an inverted light transmission Eclipse E400 microscope (5×, Nikon).

2.10. 3D free-floating gel contraction

Lattice cultures were performed in a 3D collagen-rich matrices, according to a described technique (Tomasek et al., 2002; Berton et al., 2000). Briefly, fibroblastic-keratocytes were added to bacteriological 35 mm dishes (4.5×10^4/mL) containing 0.5% FBS-medium and 100 mM NaOH immediately after the addition of type I collagen (from rat tail tendon, reconstituted in 18 mM acetic acid at a final concentration of 2 μg/mL; Berton et al., 2000) in medium alone, or in the presence of each additive alone or in combination (final pH of gels 7.2). Gel diameters were estimated in a blind fashion every day until maximum active contraction (usually 12 days) by placing the dishes on a graduated ruler over a black surface.

2.11. Statistical analysis

For each primary culture obtained from surgery room, each singular experiment was repeated three times, performed in triplicate or quadruplicate. Parametric ANOVA followed by a Tukey–Kramer post hoc analysis was used (Wilcox, 1987). A probability of ≤5% ($P < 0.05$) was presumed to reflect statistical significant difference between group mean values. The statistical package used was StatView II for PC (Abacus Concepts. Inc., Barkley, CA, USA).

3. Results

3.1. Primary fibroblastic-keratocytes express NGF, trkA^{NGFR}, and p75^{NTR}

In order to investigate the functional effect of NGF, primary cultures were incubated in the presence of FCS, and characterized for their expression of NGF, trkA^{NGFR} and p75^{NTR}. Cultured fibroblastic-keratocytes released biologically active NGF (668.25 ± 169.34 pg/mL). As detected by RT-PCR, these fibroblastic-keratocytes also synthesized NGF, trkA^{NGFR} and p75^{NTR} mRNAs (Fig. 1A). According to the molecular data, the cells expressed trkA^{NGFR} and p75^{NTR} proteins, as demonstrated by confocal and western blot analysis (Fig. 1B–E, ×40).

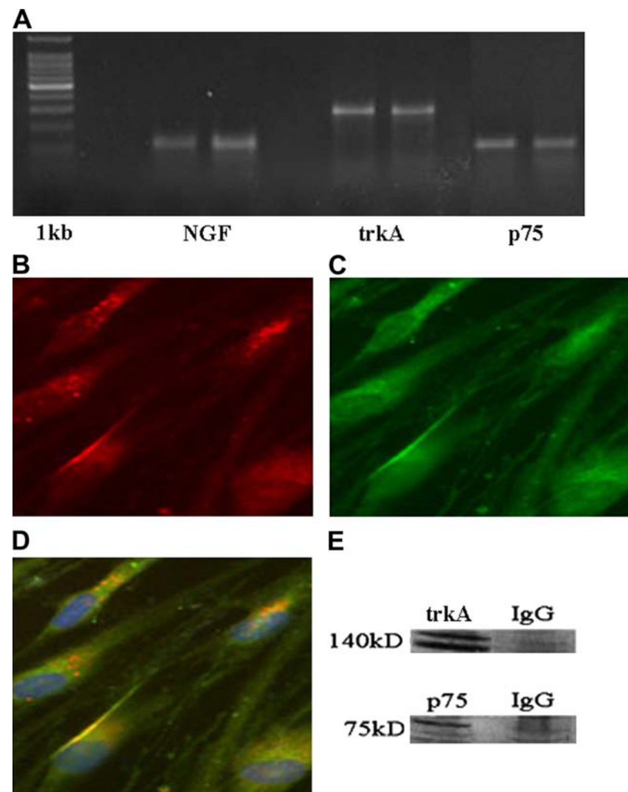

Fig. 1. NGF, trkA^{NGFR} and p75^{NTR} expression in primary fibroblastic-keratocytes. NGF, trkA^{NGFR} and p75^{NTR} mRNAs from primary human fibroblastic-keratocytes (A). The gel is representative of three experiments, where RT-PCR was performed using primers amplifying 120 bp NGF, 250 bp trkA^{NGFR} and 120 bp p75^{NTR} PCR products respectively. For confocal analysis, fibroblastic-keratocytes were cultured in 10% FCS-medium (24 h) and stained for anti-p75^{NTR} (B), anti-trkA^{NGFR} (C) and overlay (D). The nuclei were counterstained with Hoechst 332581 (blue staining). Magnification, ×400. Western blot analysis for trkA^{NGFR} and p75^{NTR} expression (E). Negative control staining, performed by incubation of samples with non-specific rabbit/goat IgG, confirmed the specificity of the signal.

3.2. NGF Induction of fibroblastic-keratocyte differentiation into myofibroblasts

In healing corneal wounds, fibroblastic-keratocytes are known to differentiate into myoFBs that express high amount of αSMA (Jester and Ho-Chang, 2003), the major protein involved in ECM contraction (Tomasek et al., 2002). According to previous reports, fibroblastic-keratocytes expressed low amount of αSMA which increased during in vitro 10% FCS-medium expansion, in the absence of any additives (from 668.25 ± 169.34 pg/mL (3rd passage) to 936.43 ± 51.52 pg/mL (5th passage), $P < 0.05$). Therefore, all experiments regarding αSMA were carried out at early generations. Structural analysis showed that the NGF treatment induced a typical fibroblast cytoskeletal reorganization, spindle-like and elongated morphology representative of myoFBs, the αSMA expressing cells (Fig. 2B; ×40). Long-term exposure to different concentrations of NGF (0–500 ng/mL) resulted in an increased expression of αSMA protein by fibroblastic-keratocytes, as a result of differentiation into myoFBs (Fig. 2A; $P < 0.01$). The maximal NGF effect on αSMA expression was found with 250 ng/mL

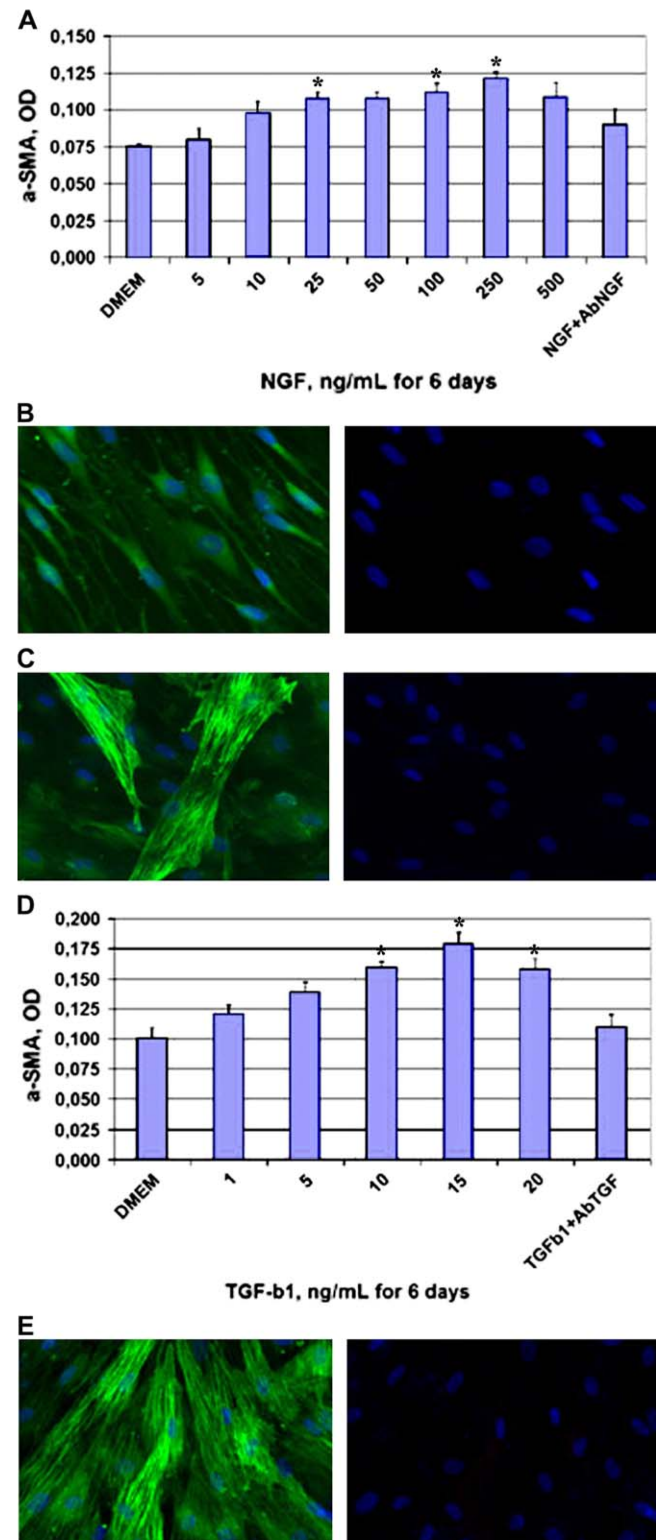

Fig. 2. Effect of NGF and TGF-β1 on expression of αSmooth Muscle Actin (αSMA) protein on Fibroblastic-keratocytes. The cells were incubated with the indicated concentrations of NGF (A) or TGF-β1 (B) and after 6 days the monolayers were immunostained for αSMA protein (cell surface-ELISA). The graph in A and D displays the effect of different concentrations of NGF (5–500 ng/mL) and TGF-β1 (1–20 ng/mL) respectively. Pre-incubations of NGF or TGF-β1 with the specific neutralizing antibodies almost completely abolished the effect. The values are expressed as O.D. (mean ± SEM) of an experiment representative of three. All P values are in comparison with medium alone (basal expression). Panel B displays the expression of αSMA under basal culture conditions; panel C displays the effect of 50 ng/mL NGF on αSMA expression; panel E displays the effect of 5 ng/mL TGF-β1 on αSMA expression. Negative controls with non-specific mouse IgG were carried out in parallel to verify the specificity of the signal. Magnification, B, C, E, ×400.

NGF ($P < 0.001$). Pre-incubation of cells with 1 μg/mL AbNGF before the addition of 250 ng/mL NGF almost abolished the effects of NGF (Fig. 2A; $P < 0.05$). According to literature data, TGF-β1 induced a significant increase in αSMA ($P < 0.001$) and myoFB differentiation in a dose-dependent fashion (Fig. 2C–D; ×40). The maximal TGF-β1 effect on αSMA expression was found with 15 ng/mL TGF-β1 ($P < 0.001$). Pre-incubation of cells with 1 μg/mL AbTGFβ before the addition of 15 ng/mL TGF completely abolished the effects of TGF-β1 (Fig. 2D; $P < 0.05$).

To confirm that the αSMA expression was due to an active synthesis of the protein, a relative real-time PCR was carried out, demonstrating an up-regulation of αSMAmRNA after NGF treatment, with a maximum at 250 ng/mL NGF (1.95-fold increase), according to the protein data (Fig. 3A). The specificity of αSMA PCR product was confirmed by the single melting curves obtained during the amplification (Fig. 3B).

3.3. Lack of NGF effect on fibroblastic-keratocyte proliferation

Since proliferation represents one of the biological markers of pro-fibrogenic activity, three different methods (Trypan blue exclusion test, [^{3}H]-thymidine incorporation and Ki67 immunocytochemistry) were employed to assess the effect of NGF on proliferation of subconfluent and confluent fibroblastic-keratocytes. Interestingly and in contrast to the well-known TGF-β1 effect observed at both 10 and 50 ng/mL (Jester and Ho-Chang, 2003), the addition of exogenous NGF (5 to 500 ng/mL) did not influence the proliferation index of fibroblastic-keratocytes, as detected by any of the techniques employed, including the [^{3}H]-thymidine incorporation assay displayed in Fig. 4A.

3.4. NGF increased migration of injured fibroblastic-keratocytes

Migration of fibroblastic-keratocytes towards the injured area is an important step in the healing process of cornea. Interestingly, the addition of NGF (10 to 250 ng/mL) induced a significant increase in the migration of these cells beyond the wound line 24 h after injury (Fig. 4B). The maximum distance of cellular migration beyond the wound line was observed with 50 ng/mL NGF,

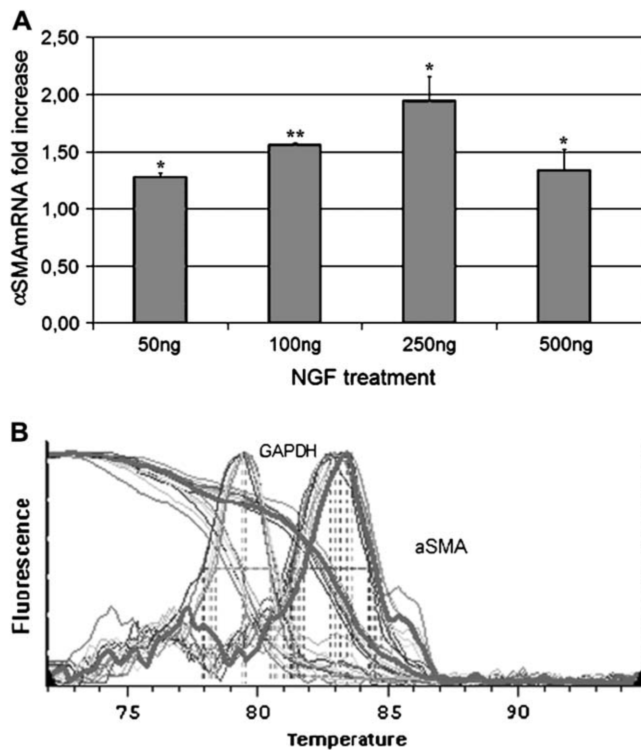

Fig. 3. Effect of NGF on αSmooth Muscle Actin (αSMA) mRNA expression in Fibroblastic-keratocytes. Total RNA from fibroblastic-keratocytes was reverse-transcribed and amplified with αSMA primers to generate a 100 bp PCR product in an Opticon 2 MJ, real time PCR machine. A: relative αSMA mRNA increase values obtained from Pfaffl program (2000); B: representative melting curve indicating that only one αSMA PCR product was amplified. Data were normalized to GAPDHmRNA levels and expressed as fold increase with respect to un-stimulated cells. ANOVA-Tukey–Kramer post hoc, $*P < 0.05$; $**P < 0.01$.

as compared to wounded fibroblastic-keratocytes in medium alone ($P < 0.01$). No significant effect on cell migration was observed with the addition of 10 ng/mL TGF-β1, according to literature data (Jester and Ho-Chang, 2003).

3.5. NGF stimulation of ECM contraction in gel lattice-embedded fibroblastic-keratocytes

Contraction of collagen, the major ECM protein, is an important event that occurs in the last stages of wound healing. NGF (50, 100 or 250 ng/mL) induced a significant increase of gel contraction by fibroblastic-keratocytes (9×10^4 cells) seeded in a three-dimensional collagen lattice ($P < 0.05$; Fig. 4C). The highest rate of matrix contraction was found at 50 ng/mL NGF ($P < 0.05$), comparable to those induced by 10 ng/mL TGF-β1, used as a positive control for contraction (Jester and Ho-Chang, 2003). Our results show a minor effect of TGF-β1 in inducing collagen contraction when compared to Jester data, but this discrepancy may reflect the different techniques used (free-floating vs. attached 3D collagen gel contraction) and different experimental conditions. Pre-incubation of cells with growth factor plus 1 μg/mL specific antibodies almost abolished the effects of both NGF (100 ng/mL) and TGF-β1 (10 ng/mL) (data not shown).

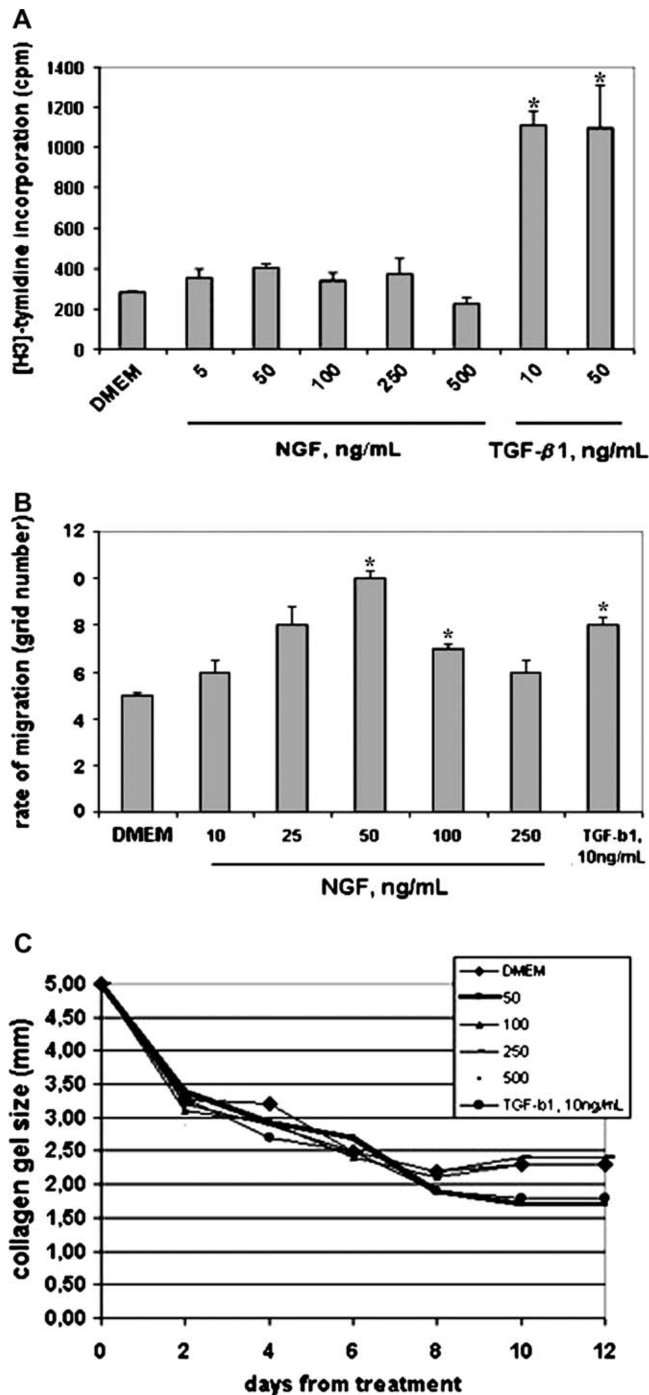

Fig. 4. Effect of NGF on keratocyte proliferation (A), migration (B) and collagen lattice contraction (C). A. Different concentrations of NGF and TGF-β1 were added to primary fibroblastic-keratocytes and their proliferation evaluated. NGF had no effect at any concentration tested, while TGF-β1 significantly increased fibroblastic-keratocyte proliferation. Data are the mean ± SEM of four experiments performed in triplicate. B. A wound line was performed on a confluent monolayer and the ability of wounded cells to migrate across the line in the presence of factors was determined. Data are number of grids, i.e., distance of cell migration (mean ± SEM). C. Collagen lattices were seeded on day 0 with 9×10^4 cells/lattice in the presence of increasing concentrations of NGF (50–500 ng/mL) or TGF-β1 (10 ng/mL). The quantitative effect after 12 days of culture in the presence of NGF is shown. The data are the mean of three similar experiments (mean ± SEM).

3.6. Lack of NGF effect on collagen type I and IV expression

Western blot analysis and cell surface ELISA showed that collagen type I and IV were not influenced by long term stimulation with NGF at any concentration tested ($P > 0.05$), while TGF-β1 induced a significant increase in both collagen types ($P < 0.05$) (data not shown).

3.7. NGF induces MMP-9 expression/activity by fibroblastic-keratocytes

According to the statement that wound-healing process comprise not only a newly synthesis of ECM, but also the expression of the tissue matrix related protease enzymes, and in particular the MMP-2 and MMP-9, named gelatinases A and B (Stamekovic, 2003; Visse and Nagase, 2003), the effect of NGF on cell gelatinolytic activity was evaluated by zymographic analysis. This study showed the presence of gelatinolytic activity in NGF treated cells, corresponding to both latent (110 kD) and active (97 kD) MMP-9 bands. In particular, NGF (50, 100, 250 or 500 ng/mL) induced a significant increase of cell gelatinolytic activity (21%, 22%, 26% and 24% respectively, as compared to control, $P < 0.05$). To investigate if this effect was related to an increase of MMP-9 synthesis, MMP-9mRNA expression was evaluated by real-time PCR. According to the functional data, a significant high expression of MMP-9mRNA after 100 ng/mL and 250 ng/mL NGF (2.16 and 2.13 fold increase respectively, $P < 0.05$), with a mild increase at 50 ng/mL NGF (1.13 fold increase, $P < 0.05$) and 500 ng/mL NGF (1.49 fold increase, $P > 0.05$), as normalized to GAPDH expression (Fig. 5A). The specificity of MMP-9 PCR product was confirmed by the presence of only one amplified product during the real-time PCR quantification (Fig. 5B). These data further indicated that NGF induced both synthesis and activation of MMP-9.

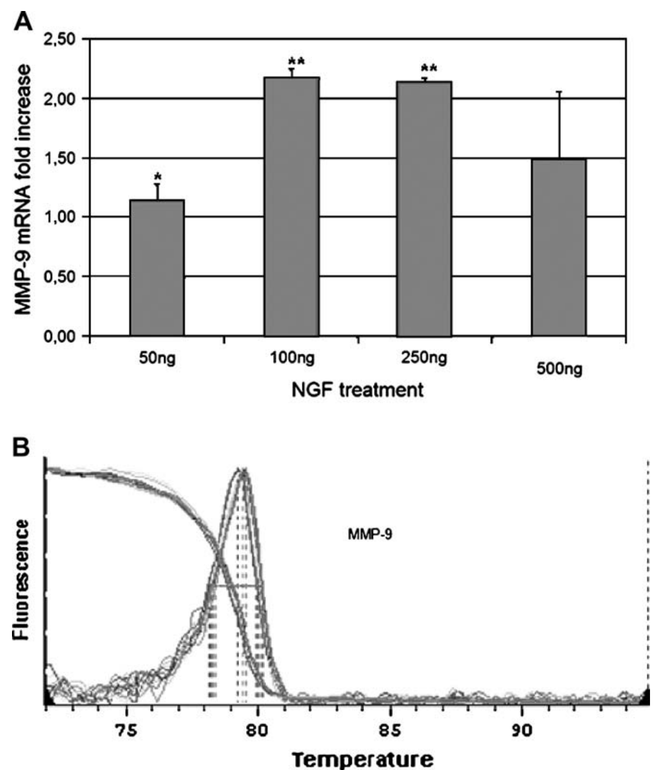

Fig. 5. Effect of NGF on MMP-9 mRNA expression in fibroblastic-keratocytes. Total RNA from fibroblastic-keratocytes was reverse-transcribed and amplified with MMP-9 primers to generate a 100 bp PCR product in an Opticon 2 MJ, real time PCR machine. A: relative MMP-9mRNA increase values obtained from Pfaffl program (2000); B: representative melting curve indicating that only one MMP-9 PCR product was amplified. Data were normalized to GAPDHmRNA levels and expressed as fold increase/decrease with respect to un-stimulated cells. ANOVA-Tukey–Kramer post hoc, $^{*}P < 0.05$; $^{**}P < 0.01$.

3.8. Reciprocal stimulation of NGF and TGF-β1 in fibroblastic-keratocytes

By investigating the effects of NGF on some pro-fibrogenic activities of fibroblastic-keratocytes in comparison to TGF-β1 effects, the question was as whether these specific effects where due to a reciprocal NGF and TGF-β1 stimulations. Therefore, to investigate if NGF stimulated TGF-β1 release, an NGF dose response curve (5 to 500 ng/mL) was performed on fibroblastic-keratocytes cultured in monolayer (Fig. 6A). NGF stimulated the release of TGF-β1 ($P < 0.05$), in a dose-dependent fashion, with a maximum effect observed at 100 ng/mL NGF. Interestingly, TGF-β1 also influenced NGF release by these cells. The addition of increasing doses of TGF-β1 (from 1 to 25 ng/mL) to monolayer induced a significant dose-response increase of NGF in the medium ($P < 0.05$; Fig. 6B), with a maximum effect observed at 15 ng/mL TGF-β1. According to biochemical data, real-time PCR analysis showed that individual 250 ng/mL NGF stimulation induced a 1.04 fold increase of TGF-β1 mRNA and 70.42 fold decrease of NGF mRNA. Moreover, the addition of 15 ng/mL TGF-β1 induced a consistent 63.03 fold increase of NGF mRNA and a mild 1.61 fold increase of TGF-β1 mRNA. The addition of AbNGF to TGF-β1 or the AbTGFβ to NGF reduced the expression of both NGF and TGF-β1 mRNAs (4.69 and 6.82 fold-decrease and 2.39 and 5.70 fold-decrease, respectively).

To demonstrate the specificity of both growth factors' effects on the myoFBs differentiation (αSMA expression), monolayers were incubated with 250 ng/mL NGF alone or in the presence of AbTGFβ (250 ng/mL), with 15 ng/mL TGF-β1 alone or in the presence of AbNGF (500 ng/mL) or with a mixture of both growth factors (250 ng/mL NGF and 15 ng/mL TGF-β1). cs-ELISA showed that AbNGF as well as AbTGFβ were not able to inhibit the effects of TGF-β1 and NGF respectively, as reported in Fig. 6C. By the way, no additive or synergic effects were observed when the factors were added as a mixture (Fig. 6C). In particular, neutralizing antiTGFβ antibodies (1, 10 and 25 μg/mL) showed no significant effect on NGF induced αSMA protein levels, as observed by densitometric analysis in western blotting assay specific for αSMA (Fig. 6D).

In order to investigate at the molecular level the possible contribution of NGF and TGF-β1 to αSMA expression,

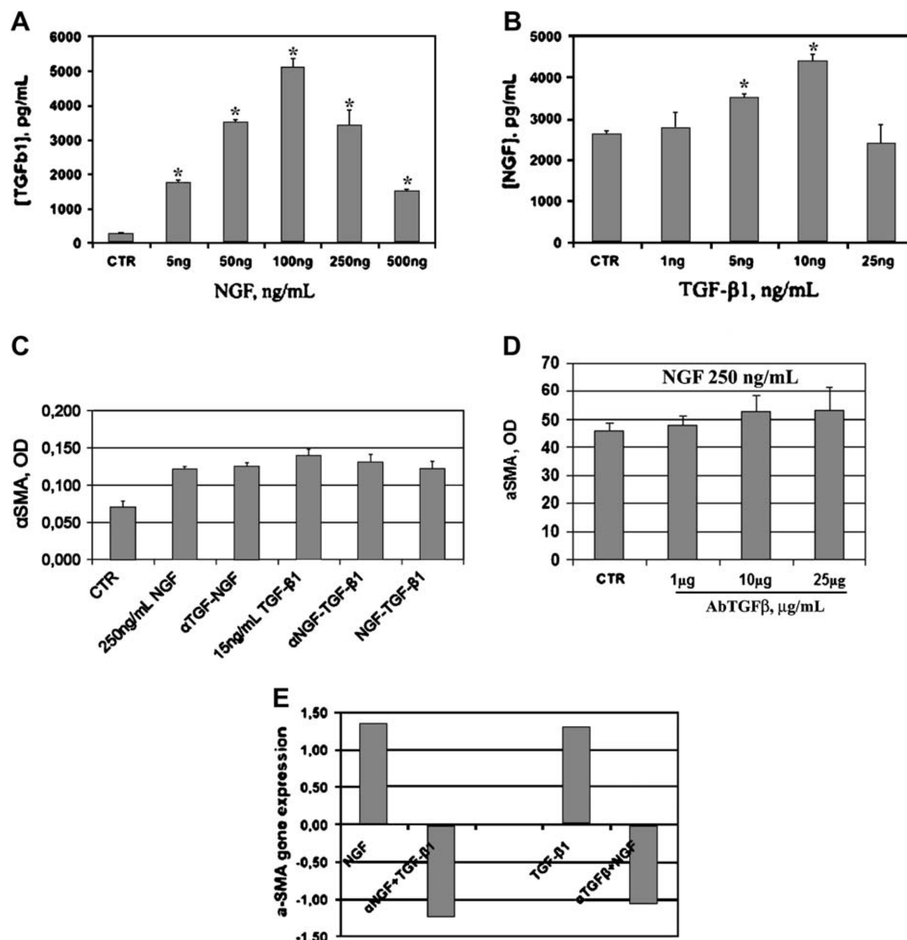

Fig. 6. Reciprocally expression of TGF-β1 and NGF proteins/mRNAs and in fibroblastic-keratocytes. The ELISA analysis of TGF-β1 (A) and NGF (B) release in conditioned media of stimulated fibroblastic-keratocytes is displayed. Cell surface ELISA analysis for αSMA expression by NGF and TGFβ1 alone, together or in the presence of AbTGFβ (250 ng/mL) or AbNGF (500 ng/mL) respectively, as compared to control (untreated cells) (C). Densitometric analysis (Western blot) of αSMA expression by NGF in the presence of increasing neutralizing antiTGFβ antibodies (1, 10, 25 µg/mL) (D). Values are mean ± SEM of 3 experiments performed in triplicate; error bars indicate SEM. The molecular analysis of αSMAmRNAs expression by NGF and TGF-β1 stimulated monolayers, is displayed in E. Data are expressed as real C(t), and differences are showed in the graphics as the expression ratio of normalized target gene, according to the software results. The graph is representative of 3 different experiments performed in triplicate; error bars indicate SEM. Upon standard conditions, only one PCR product was amplified.

a specific real-time experiment was carried out. According to this study, the addition of neutralizing AbTGF to 250 ng/mL NGF resulted in a decreased αSMA mRNA expression (1.22 fold decrease; Fig. 6E). Likewise, the addition of AbNGFβ to 15 ng/mL TGF-β1 resolved in a decreased αSMA mRNA expression (1.06 fold decrease; Fig. 6E).

4. Discussion

The present study was aimed to investigate NGF effects on human fibroblastic-keratocyte functions, both at the biochemical and molecular levels. In brief, NGF was found to stimulate fibroblastic-keratocyte differentiation into αSMA expressing cells (myoFBs), their 3D-collagen contraction, and their MMP-9 expression, without affecting neither proliferation or collagen production. These effects were probably mediated by trkA^{NGFR} and p75^{NTR} receptors, both expressed by unstimulated fibroblastic-keratocytes.

NGF is a pleiotropic factor playing a crucial character in the patho-physiology of nervous, endocrine, immune and visual systems (Lambiase et al., 2004; Micera et al., 2005) and showing a precise role in wound healing and tissue repair, after traumatic muscle injury and in diabetic conditions, as previously reported (Li et al., 1980; Schultz et al., 1994; Matsuda et al., 1998; Kasemkijwattana et al., 2000). In the ocular surface, NGF, trkA^{NGFR} and p75^{NTR} have been observed during normal and pathological conditions, mainly expressed by epithelial and stromal cells populating both conjunctiva and cornea (Lambiase et al., 1998a; You et al., 2000). Interestingly, the topical NGF eye-drop treatment restored stromal and epithelial integrity in patients with neurotrophic or autoimmune corneal ulcers, highlighting the crucial role of NGF in corneal healing process (Lambiase et al., 1998b, 2000b). As a partial explanation, it has been prospected that the NGF healing result might be due to a direct effect of NGF on epithelial cells, which are known to express trkA^{NGFR} and to proliferate/differentiate upon NGF stimulation (You et al., 2000; Lambiase et al., 2000b). Corneal

healing, minutely regulated by growth factors, cytokines and neuropeptides, proceeds through an early phase of keratocyte activation/proliferation, cell migration to the area of injury and myoFB differentiation, followed by further proliferation and ECM production (Wilson et al., 2001; Schultz et al., 1994; Ye and Azar, 1998). Several studies have shown a crucial role of TGF-β1 in promoting keratocyte proliferation and differentiation (Jester et al., 1999; Jester and Ho-Chang, 2003), according to TGF-β1 beneficial effects, other growth factors and cytokines, might cooperate in coordinating the entire wound-healing process (Wilson et al., 2001; Schultz et al., 1994; Ye and Azar, 1998).

Since fibroblastic-keratocytes and myoFBs represent the main target/effector cells of the entire corneal healing process (Jester and Ho-Chang, 2003), and since NGF treatment induced corneal ulcer recovery (Lambiase et al., 1998b), the present study was aimed at investigating if a direct effect of NGF on this specific keratocyte phenotype was at least in part responsible for the proper wound-healing effect observed in human cornea.

The simultaneous expression of NGF, $trkA^{NGFR}$ and $p75^{NTR}$ on fibroblastic-keratocytes clearly indicates the existence of a physiological NGF autocrine/paracrine pathway. The effect of NGF in promoting myoFB differentiation, as observed in monolayers and confirmed in contracting 3D collagen matrix, clearly indicate that NGF theaters critical involvement in wound closure by means of keratocyte stimulation, an effect familiar to TGF-β1.

NGF, but not TGF-β1, significantly enhanced keratocyte migration beyond a wound line (after 24 h from plating), highlighting the individual effect of NGF as a chemo-attractant factor, responsible for the migration of fibroblastic-keratocytes to the wounded area (Andresen et al., 1997). According to the observation that αSMA expression occurred at least after 2 days (and reach the maximum after 6 days) of culturing in the presence of NGF, it is likely to hypothesize that the migrating cells are still fibroblastic-keratocytes. This NGF effect was previously reported in granulocytes and lymphocytes (Boyle et al., 1985) taking part in the early stages of healing, and more recently, in lung, skin and conjunctival fibroblasts (Micera et al., 2001, 2005). This NGF induced keratocyte migration strongly indicates a essential role of NGF in corneal healing, where cell recruitment to the damaged area is an essential and prime step to attempt recovery. In contrast to migration results, NGF did not induce fibroblastic-keratocyte proliferation or collagen production, according to skin, lung and conjunctiva fibroblast results (Micera et al., 2001, 2005), two features typical of TGF-β1 (Jester and Ho-Chang, 2003).

On the other side, increased proteolytic activity is also a common response to injury (Tomasek et al., 2002), and one class of MMPs, taking actively part in this process, is represented by the neutral proteases MMP-2 and MMP-9, also named respectively gelatinase A and B (Visse and Nagase, 2003; Ye and Azar, 1998). Previous studies have provided evidences that NGF, via MAP kinase activities (Erks 1 and 2), can trigger the expression of MMP-9 in PC12 and smooth muscle cells (Shubayen and Myers, 2004; Faisal Khan et al., 2002). Because MMPs regulate the ECM during corneal wound-healing (Visse and Nagase, 2003), being important participants in the process of connective tissue remodeling, in this study the possible NGF modulation of MMP-9 expression by fibroblastic-keratocytes was also investigated. Zymograghy analysis identified NGF as a possible inducer of MMP-9 activity in fibroblastic-keratocytes, as observed by the gelatinolytic band clearly visible upon NGF stimulation. This NGF-induced MMP-9 expression would imply that during the corneal healing process, NGF might directly facilitate the migration of cells towards the site of injury not only by inducing active migration but also by degrading the ECM via MMP-9 activity, according to previous reports (Khan et al., 2002).

The observation that NGF and TGF-β1 could regulate their reciprocal molecular/biochemical expression in fibroblastic-keratocytes, would imply a cooperating NGF/TGF-β1 effect on wound-healing. Consistent with the fact that several cytokines and growth factors are released in the injured area (Visse and Nagase, 2003; Jester and Ho-Chang, 2003), and exert specific, complementary and sometimes opposite effects. In order to elucidate NGF action might be not straight but mediated by TGF-β1, blocking experiments were carried out. The blocking experiments clearly demonstrated that NGF effects on myoFB differentiation are independent by TGF-β1. In addition, neutralization of NGF failed to decrease the αSMA differentiation mediated by TGF-β1. One possible explanation lay in the fact that both growth factors are straight and sufficient for αSMA differentiation. The observation that neutralization of NGF induced a decrease of αSMA mRNA expression by TGF-β1, as well as vice-versa, is apparently in contrast with the αSMA protein data, even though it may suggest the existence of a post-transcriptional regulation of αSMA. Few data on the NGF-induction via TGF-β1 and vice-versa are reported in literature but several aspects of NGF/TGF-β1 interaction appear actually obscure (Lutz et al., 2004).

Taken together, all these data show a consistent and selective contribution of NGF in stimulating healing activities in cultured fibroblastic-keratocytes, along with a TGF-β1 cooperation, that cannot be excluded.

Accordingly, during the corneal healing process, NGF might act in synergy or as a complement to TGF-β1, and both growth factors can reciprocally influence their release. These findings in vitro would provide additional information on the possible cellular mechanisms of NGF, besides the well-known TGF-β1 implication, in promoting corneal healing.

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