TESI DOCTORAL

LES CÈL·LULES PROGENITORES ENDOTELIALS COM A TRACTAMENT NEUROREPARADOR ANGIO-VASCULOGÈNIC EN LA ISQUÈMIA CEREBRAL MODULAT PER LA METAL·LOPROTEÏNASA DE MATRIU 9 (MMP-9)

ANNA MORANCHO RETANA

FACULTAT DE MEDICINA
DEPARTAMENT DE MEDICINA. UNIVERSITAT AUTÒNOMA DE BARCELONA

Directors:
DRA. ANNA ROSELL NOVEL
DR. JOAN MONTANER VILLALONGA

RESUM

Avui dia la patologia isquèmica cerebral, amb una fisiopatologia molt complexa, segueix sent una de les causes més importants de mort i incapacitat al món. Actualment l'únic tractament possible són les teràpies trombolítiques amb l'activador del plasminogen de teixit (rt-PA) , que només s'administren en la fase hiperaguda dels ictus isquèmics (<4,5 hores). És per tant necessari investigar noves teràpies aplicables després de la fase hiperaguda per poder augmentar el nombre de pacients tractats. La neuroreparació és una estratègia terapèutica que permet restablir la circulació cerebral i promoure la neuroregeneració. Per a assolir aquests objectius cal potenciar l'angiogènesi i la neurogènesi en el cervell isquèmic. L'objectiu d'aquesta tesi és estudiar el potencial neuroreparador de les cèl·lules progenitores endotelials (EPCs) i el rol de la MMP-9 en la seva modulació.

Utilitzant un model d'isquèmia cerebral permanent en ratolí es va estudiar el potencial terapèutic de les EPCs així com dels factors secretats per aquestes. Els resultats mostren la potenciació de les respostes angiogèniques i neurogèniques endògenes en els animals que reben els citats tractaments demostrant per primer cop en un model d'isquèmia cerebral el potencial terapèutic dels factors de creixement que secreten les EPCs, oferint una eina per a teràpies pro-angiogèniques autòlogues basades en factors de creixement i lliures de cèl·lules.

Atès que sabem que les metal·loproteïnases de matriu (MMPs) són necessàries per la remodelació extracel·lular que es dóna durant els processos d'angiogènesi i neurogènesi, aquesta tesi aprofundeix en el rol de la MMP-9 com a molècula clau reguladora en les teràpies pro-angiogèniques després de la isquèmia cerebral. Alhora també s'ha estudiat el rol de la isquèmia en l'activació i mobilització de les EPCs. Els resultats d'aquesta tesi mostren que la isquèmia cerebral estimula l'alliberament de les EPCs a la circulació. Per altra banda també hem demostrat com la deficiència de MMP-9 produeix una disminució de les EPCs circulants en animals control, que es reverteix després de l'estímul isquèmic. També s'han realitzat estudis de tubulogènesi *in vitro*, que demostren un augment de les capacitats angiogèniques en les cèl·lules obtingudes d'animals isquèmics comparat amb no isquèmics així com una disminució d'aquestes en cèl·lules d'animals deficients en MMP-9. La inhibició de les MMPs, i específicament la MMP-9, en EPCs humanes mostren els mateixos resultats, confirmant el rol d'aquesta metal·loproteïnasa en les capacitats angio-vasculogèniques *in vitro* de les EPCs.

Finalment, s'ha posat a punt un model d'isquèmia cerebral transitòria en ratolí, per oclusió distal de l'artèria cerebral mitja mitjançant la seva compressió. Aquest model, amb un infart cortical ben delimitat i amb l'existència de la reperfusió del teixit, presenta una major homologia amb la patologia humana i ens permetrà continuar amb els estudis de neuroreparació en situacions de reperfusió.

ABSTRACT

Nowadays acute ischemic brain disease, which has a complex pathophysiology, remains one of the most important causes of death and disability worldwide. Currently the only possible treatment is the thrombolytic therapy with tissue plasminogen activator (rt-PA), that can be administered only in the hyperacute phase of ischemic stroke (<4.5 hours). Therefore, it is necessary to investigate new therapies that could be applicable after the hyperacute phase to increase the number of treated patients. Neurorepair therapies are therapeutic approaches that allow restoring cerebral circulation and promoting neuroregeneration. To achieve these objectives it is necessary to enhance angiogenesis and neurogenesis in the ischemic brain. The aim of this thesis is to study the neuropair potential of endothelial progenitor cells (EPCS) and the role of MMP-9 in its modulation.

Using a model of permanent cerebral ischemia in mice, we studied the therapeutic potential of EPCs and their secreted factors. The results show an increase of angiogenesis in animals receiving the aforementioned treatments, demonstrating for the first time the therapeutic potential of growth factors secreted by EPCs in a model of cerebral ischemia and providing a tool for autologous pro-angiogenic cell-free therapies.

Since it is known that matrix metalloproteinases (MMPs) are required for the extracellular remodeling that occurs during angiogenesis and neurogenesis, this thesis explores the role of MMP-9 as a key molecule in the regulation of pro-angiogenic therapies after cerebral ischemia. In addition, the role of ischemia in the activation and mobilization of the EPCs has also been studied. The results of this thesis show that cerebral ischemia stimulates the release of EPCs to circulation. Furthermore we have also demonstrated that MMP-9 deficiency causes a decrease in the number of circulating EPCs in control animals, which is reversed after the ischemic insult. *In vitro* tubulogenesis studies showed an increased angiogenic capacity in cells obtained from ischemic animals compared with non-ischemic as well as a decrease of these abilities in MMP-9 deficient animals. The pharmacological inhibition of MMPs, specifically MMP-9 in human EPCs showed the same results, confirming the role of this metalloproteinase in the *in vitro* angio-vasculogenic abilities of EPCs.

Finally, we have developed a model of transient cerebral ischemia in mice by occlusion of the distal middle cerebral artery by compression. This model, with a well-defined cortical infarct and the presence of tissue reperfusion, has a greater homology with human disease and allows us to continue the neurorepair studies in the presence of reperfusion.

ÍNDEX DE CONTINGUTS

ABREVIATURES

ACE: de l’anglès *angiotensin-converting enzyme*

ACM: artèria cerebral mitja

AIT: atac isquèmic transitori

ATP: de l’anglès *adenosine triphosphate*

AMPA: de l’anglès *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid*

Ang-1: angiopoietina 1

BDNF: de l’anglès *brain-derived neurotrophic factor*

BHE: barrera hematoencefàlica

CACs: de l’anglès *circulating angiogenic cells*

CD: de l’anglès *cluster of differentiation*

CXCR4: de l’anglès *Chemokine (C-X-C motif) receptor 4*

ECFCs: de l’anglès *endothelial colony forming cells*

EPC: de l’anglès *endothelial progenitor cell*

EPO: eritropoetina

FGF: de l’anglès *fibroblast growth factor*

G-CSF: de l’anglès *granulocyte colony stimulating factor*

HGF: de l’anglès *fibroblast growth factor*

IL: de l’anglès *interleukin*

iPS: de l’anglès *induced pluripotent stem cells*

KO: de l’anglès *knock-out*

MCP-1: de l’anglès *monocyte chemotactic protein-1*

MMPs: de l’anglès *matrix metalloproteinases*

NGF: de l’anglès *nerve growth factor*

NMDA: de l’anglès *N-methyl-D-aspartatic acid*

OECs: de l’anglès *outgrowth endothelial cells*

PDGF: de l’anglès *platelet-derived growth factor*

rt-PA: de l’anglès *recombinant tissular plasminogen activator*

SDF-1: de l’anglès *stromal derived factor 1*

TGF-β: de l’anglès *transforming growth factor beta*

Tie2: receptor de l'angiopoietina 1 i 2

TIMP: de l'anglès *tissue inhibitor of metalloproteinase*

TNF: de l'anglès *tumor necrosis factor*

TSP-1: de l'angles *thrombospondin 1*

TSP-2: de l'angles *thrombospondin 2*

VEGF: de l'anglès *vascular endothelial growth factor*

VEGFR-2: de l'anglès *vascular endothelial growth factor receptor 2,* també conegut com KDR o Flk-1

vWF: de l'anglès *von Willenbrand factor*

WT: de l'anglès *wild-type*

1. INTRODUCCIÓ

1.1 L' ictus

L'ictus o patologia cerebrovascular aguda és un trastorn circulatori que es produeix com a resultat d'una alteració del flux sanguini cerebral de forma transitòria o permanent per una isquèmia (oclusió del vas) o una hemorràgia (trencament del vas d'una o més arteries cerebrals) desencadenant un procés patològic. Com a resultat de la manca de reg sanguini al cervell poden aparèixer tota una sèrie de símptomes neurològics com l'afàsia (trastorn de la parla), hemiparèsia (afectació de la capacitat motora d'un costat del cos), pèrdua de visió, vertigen o trastorns del llenguatge, entre d'altres. La seva aparició dependrà de la zona de l'encèfal que estigui afectada. Aquests dèficits es podran recuperar o no segons l'evolució clínica del pacient, on el temps d'oclusió jugarà un paper fonamental, i del tipus d'infart (1).

L'ictus continua sent un dels trastorns neurològics més devastadors, representant prop de 5,5 milions de morts anuals arreu del món i uns altres 5 milions de supervivents amb discapacitats neurològiques (dades obtingudes de la World Health Organitzation, www.who.org). En tractar-se d'una malaltia associada a l'envelliment, s'espera que la seva incidència segueixi creixent ja que la població mundial major de 65 anys continua augmentant en aproximadament 9 milions de persones l'any (2). A l'estat espanyol cada any es produeixen uns 200 casos per cada 100.000 habitants i representen la primera causa de mort en les dones, la segona en els homes i la primera causa de discapacitat adquirida en la vida adulta (3). Per tant, té un impacte econòmic molt elevat ja que una gran part dels pacients requeriran algun tipus d'assistència en el seu dia a dia.

Els factors de risc per ictus poden ser àmpliament classificats com a modificables o fixes. Alguns factors de risc modificables (com la hipertensió, la diabetis, les dislipèmies, la obesitat o el tabaquisme) són comuns i proporcionen oportunitats per modificar el risc en un gran nombre de persones. Altres factors de risc, com la fibril·lació auricular i els atacs isquèmics transitoris, són menys freqüents i més específics que els factors de risc comuns (4). A més, també hi ha factors genètics que determinen una major susceptibilitat a patir un ictus. Alguns factors de risc genètic es presenten com a malalties monogèniques (malaltia de Fabry o el CADASIL) però en la majoria de casos la relació genètica de la malaltia és resultat de l'efecte multiplicador de diferents al·lels patogènics, cadascun dels quals confereix un lleuger augment del risc (5). Els factors de risc genètic així com el gènere o l'edat són factors de risc no modificables.

1.1.1 Els subtipus d'ictus

Els ictus es classifiquen en diferents subtipus tenint en compte criteris clínics, topogràfics, patogènics i diagnòstics. En general els ictus es poden dividir en dos grans grups segons el seu origen: isquèmics o hemorràgics (**Figura 1**).

Els ictus isquèmics representen entre el 80 i el 85% de tots els ictus. Es produeixen majoritàriament com a conseqüència de l'oclusió d'una artèria per un mecanisme trombòtic (plaques d'ateroma al lumen del vas) o embolic (produït per coàguls). La disminució de l'aportació sanguínia cerebral pot ser total (isquèmia global) o parcial (isquèmia focal). La isquèmia cerebral focal, la més comú, es divideix segons la duració de l'episodi en: atac isquèmic transitori (AIT, un episodi d'inici agut i etiologia isquèmica de disfunció neurològica focal amb una duració menor a les 24h, que generalment dura entre 1 i 2 hores) o infart cerebral (6).

Els ictus hemorràgics o hemorràgies cerebrals es produeixen degut al trencament d'un vas sanguini i representen un 15-20% dels ictus. Es classifiquen en hematomes intracerebrals (extravasació de sang cap al parènquima cerebral) o hemorràgies subaracnoïdals (extravasació de sang directament a l'espai subaracnoïdal).

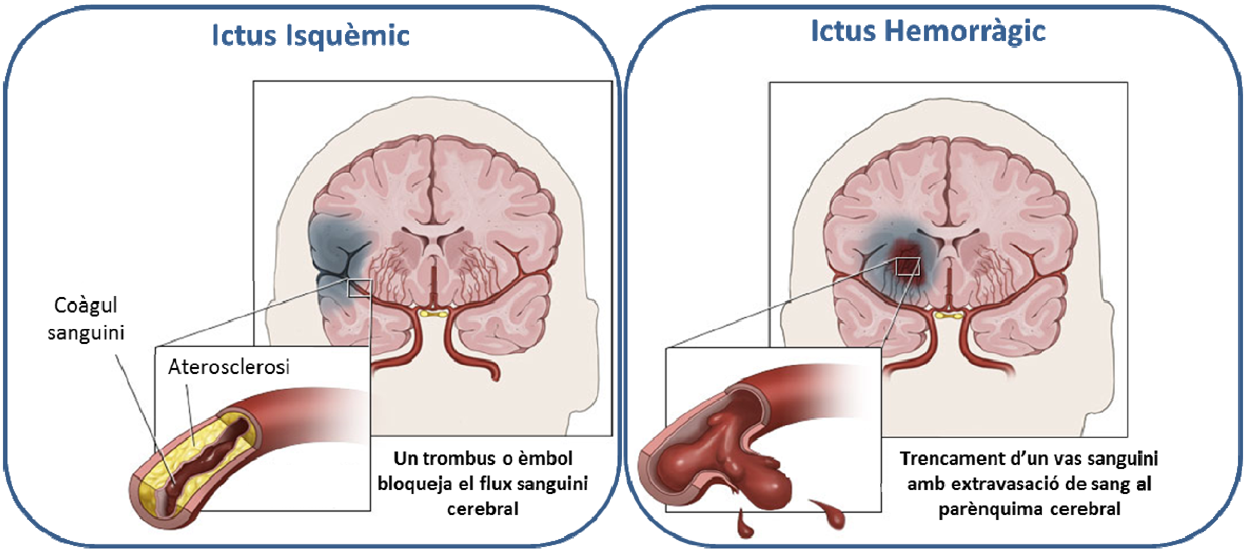

Figura 1. Esquema dels dos principals subtipus d'ictus: isquèmics i hemorràgics. (Adaptat de The Johns Hopkins Health Library.)

Els ictus isquèmics i hemorràgics es poden classificar a diferents nivells, segons la seva duració i extensió, l'àrea cerebral afectada, la localització de la lesió o la seva etiologia (**Figura 2**).

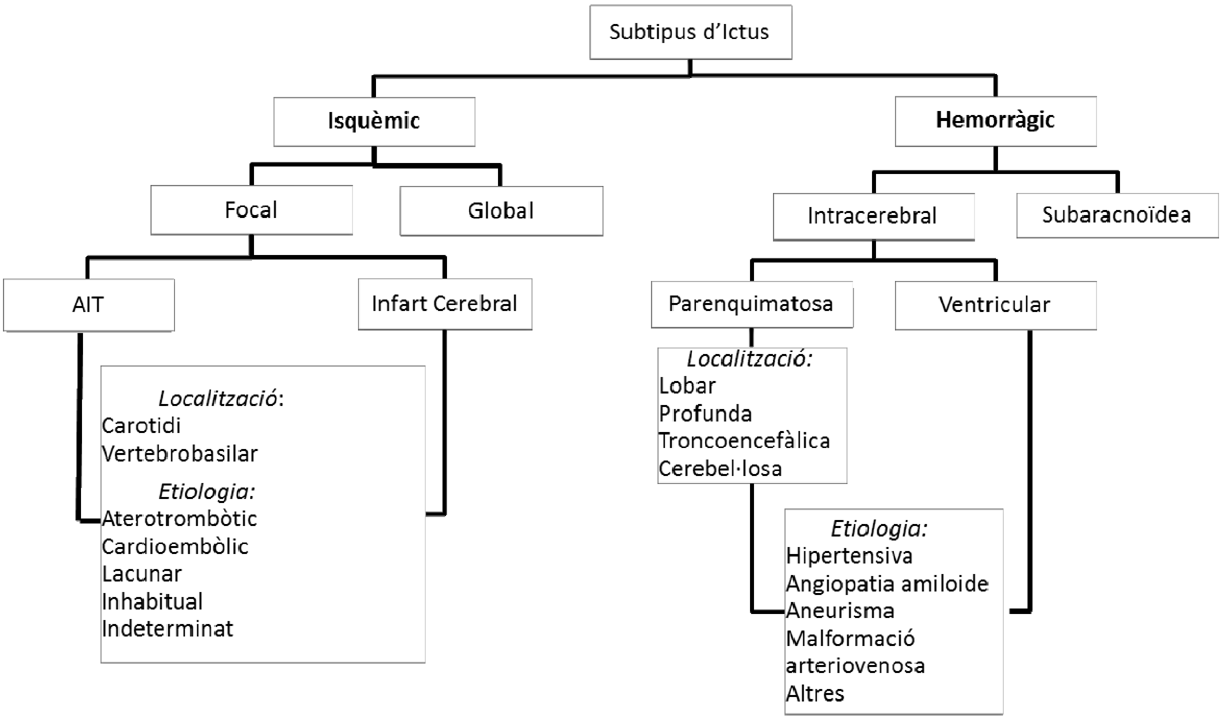

Figura 2. Classificació dels subtipus d'ictus. (Adaptada de Díez-Tejedor et al., 2001 i Arboix et al., 2006.)(1,7)

La classificació etiològica més utilitzada actualment són els criteris TOAST (*Trial of Org 10172 in Acute Stroke Treatment*), que diferencia 5 subgrups d'ictus isquèmics (1,8). Els dos subtipus més freqüents són els aterotrombòtics, deguts a una estenosi d'una artèria o la ruptura d'una placa d'ateroma que arriba al cervell en forma de trombus, i els cardioembòlics, produïts per l'obstrucció d'una artèria per material embòlic d'origen cardíac. Menys freqüents són els ictus lacunars, patologies de vas petit que produeixen infarts de mida petita (<1.5 cm de diàmetre) i els ictus de causa inhabitual, que se solen produir per malalties sistèmiques com per exemple infeccions, alteracions de la coagulació, neoplàsies o aneurismes. Tot i els avenços en les tècniques de diagnòstic no s'arriba a determinar la causa d'alguns infarts, que es classifiquen com a ictus isquèmics d'origen indeterminat.

Respecte al pronòstic, l'estat neurològic dels pacients d'ictus s'avalua a partir d'escales objectives que mesuren la gravetat del dany produït per la lesió. L'escala més utilitzada és la NIHSS (*National Institutes of Health Stroke Scale*), que amb una puntuació de 0 a 42 punts de menys a més gravetat permet valorar el dèficit neurològic i la millora o empitjorament a curt termini (dies, setmanes) (9). Una variació en el temps menor de 4 punts es considera estabilitat del pacient, un increment de 4 punts o més indica empitjorament i una baixada de 4 o mes punts indica milloria neurològica.

1.1.2 La fisiopatologia isquèmica

La conseqüència immediata de l'oclusió d'una artèria cerebral, ja sigui per un coàgul, una trombosi o una hipoperfusió sistèmica, és la restricció del flux cerebral sanguini. El resultat és una aportació d'oxigen i glucosa insuficient per a mantenir l'homeòstasi cel·lular. Això provoca múltiples processos que condueixen a la mort cel·lular: excitotoxicitat, acidosi i desequilibri iònic, estrès oxidatiu, inflamació, apoptosi i despolarització neuronal (10)(**Figura 3**).

Al votant de la zona infartada pot existir un flux sanguini residual suficient per a mantenir la viabilitat cel·lular durant un temps determinat. En aquesta zona anomenada penombra isquèmica, les cèl·lules tot i mantenir-se viables es troben metabòlicament inactives (11). La penombra és susceptible de recuperació si es recupera l'aportació de flux sanguini i per aquest motiu és la diana terapèutica més rellevant en la fase hiperaguda. El destí del teixit en zona de penombra depèn de la circulació col·lateral, el grau d'isquèmia i el temps fins la reperfusió. A la zona de penombra isquèmica la mort cel·lular és més lenta i es produeix per mecanismes de mort cel·lular activa com l'apoptosi. Si la situació d'hipoperfusió continua, la funció cel·lular alterada per la cascada isquèmica acaba provocant la pèrdua irreversible de la viabilitat cel·lular (12,13).

A l'anomenat *core* (centre) del territori isquèmic, on el flux sanguini està més restringit, la mort cel·lular es produeix per necrosi i processos excitotòxics durant els primers minuts. La reducció de l'ATP, conseqüència de l'esgotament de les reserves energètiques cel·lulars i la fallida metabòlica, provoca la despolarització de la membrana cel·lular de neurones i glia i l'alteració de la seva permeabilitat pel bloqueig de les bombes iòniques dependents d'ATP. El potencial de repòs de la membrana plasmàtica no es pot mantenir, provocant l'augment de les concentracions intracel·lulars de sodi (Na^+) i calci (Ca^{2+}) i el potassi extracel·lular (K^+). La conseqüent despolarització de la membrana ocasiona l'augment de l'alliberació de glutamat i altres aminoàcids excitadors activant els processos excitotòxics (10). L'augment en la concentració de glutamat sinàptic activa els receptors de N-metil-D-aspartat (NMDA), d'àcid amino-3-hidroxi-5-metil-4-isoxazol propiònic (AMPA) i els receptors metabotròpics de la neurona post-sinàptica, provocant l'entrada de calci a la cèl·lula i incrementant encara més la sobrecàrrega de calci intracel·lular. Com a resultat es produeix un major influx de Na^+ i Cl^- a les neurones i un eflux de K^+, provocant l'entrada passiva d'aigua i resultant en edema cel·lular per trencament de l'equilibri osmòtic.

El calci, a més, activa diferents enzims lítics com proteases, lipases i endonucleases, facilita la síntesi d'òxid nítric i radicals lliures derivats i desacobla la fosforilació oxidativa (14,15).

El calci, també activa altres vies de senyalització intracel·lular que comporten l'expressió a nivell local de citocines com el factor necròtic tumoral (TNF) o la interleucina 1β (IL-1β). Aquestes citocines estimulen l'alliberació d'altres citocines com la IL-6 i la IL-8, quimiocines i molècules d'adhesió. La sobreexpressió de les molècules d'adhesió a les cèl·lules endotelials de la barrera hematoencefàlica (BHE) indueix la infiltració de neutròfils. La infiltració d'aquestes cèl·lules incrementa el dany tissular en la fase aguda estimulant l'alliberament de substàncies vasoconstrictores i enzims proteolítics que trenquen la BHE i permeten el flux d'aigua i eritròcits, i per tant contribueixen a la creació de l'edema cerebral i la transformació hemorràgica (15-17).

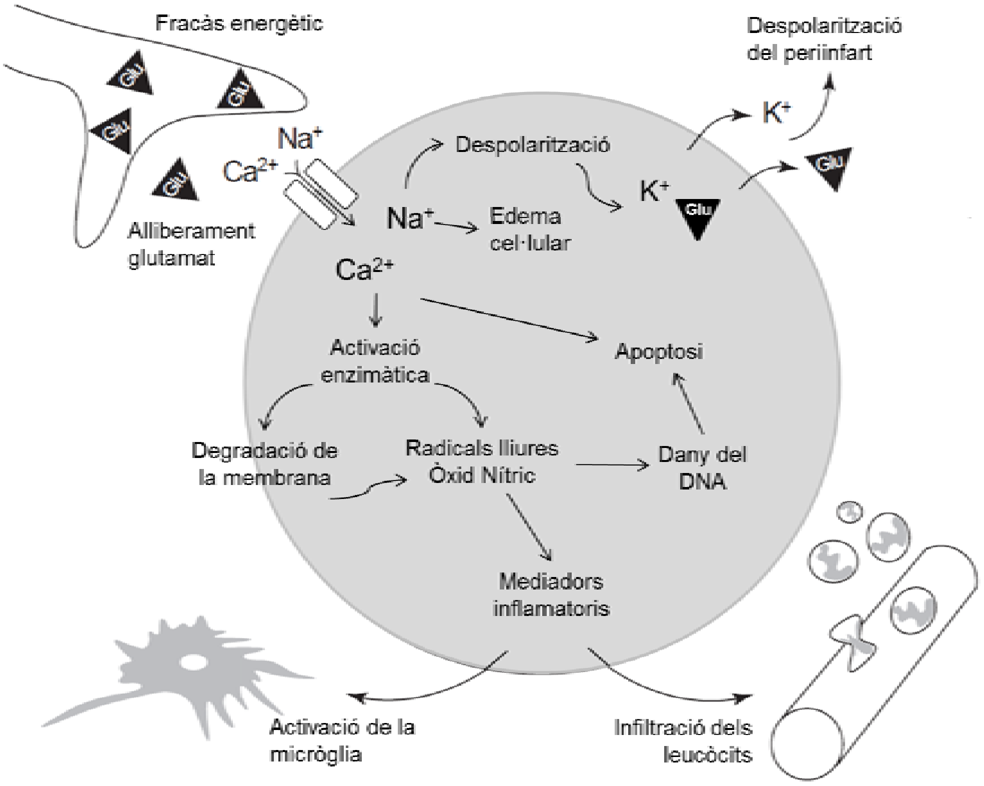

Figura 3. Resum dels processos fisiopatològics que tenen lloc en el teixit cerebral isquèmic. (Adaptat de Dirnagl et al., 1999)(18).

1.2 Tractaments i estratègies terapèutiques en l'ictus isquèmic

L'ictus és una malaltia amb un gran impacte epidemiològic i econòmic, que demanda el desenvolupament d'una estratègia de tractament efectiva. Les teràpies actuals durant la fase aguda es basen principalment en 4 pilars: la recanalització arterial per estratègies de reperfusió, la prevenció i tractament de complicacions secundàries, la neuroprotecció dirigida a dianes cel·lulars i metabòliques d'activació primerenca i la modulació de la resposta inflamatòria secundària. En canvi, en la fase subaguda de la malaltia, les teràpies neuroreparadores es basen en la potenciació dels processos cerebrals de neuroreparació amb l'objectiu de recuperar la funció cerebral i reduir el dèficit neurològic.

1.2.1 Fàrmacs trombolítics

La funció dels fàrmacs trombolítics és la dissolució del coàgul de fibrina, mitjançant la conversió del plasminogen en plasmina. Existeixen diferents fàrmacs amb aquest mecanisme d'acció, però l'únic tractament aprovat actualment per a l'ictus isquèmic és l'activador recombinant del plasminogen tissular (rt-PA) durant les 4.5 primeres hores des de l'inici dels símptomes (19). L'administració endovenosa de rt-PA és el tractament recomanat en la majoria del pacients per la seva eficàcia, però té una estreta finestra terapèutica i uns estrictes criteris d'inclusió que fan que només al voltant d'un 5% dels pacients se'n puguin beneficiar. A més, un 2-10% dels pacients tractats amb rt-PA pateixen efectes adversos com transformacions hemorràgiques que s'associen amb un augment de la mortalitat (20,21).

Avui en dia s'està avaluant la combinació dels fàrmacs trombolítics amb la trombectomia intraarterial. La trombectomia intraarterial utilitza dispositius mecànics per a l'extracció o disrupció del coàgul. Els dispositius d'extracció o recuperació del coàgul capturen o aspiren el coàgul, traient-lo de la circulació cerebral. Aquests dispositius han demostrat augmentar la taxa de recanalització i la millora del dèficit neurològic (22). Tot i que aquests mecanismes han demostrat la seva efectivitat en la recanalització, un recent estudi multicèntric ha descrit que els diferents mecanismes de teràpia endovascular (tant la trombectomia com el tractament trombolític endovascular) no serien més efectius que el rt-PA intraarterial en termes de millora funcional (23). Actualment s’estan realitzant nous assaigs clínics amb dispositius més eficaços, que podrien demostrar la utilitat de la teràpia endovascular (24).

1.2.2 La neuroprotecció

La finalitat dels tractaments neuroprotectors és interferir en la cascada isquèmica, bloquejant molècules clau dels diferents mecanismes de dany cerebral i prevenint la mort cel·lular en la penombra isquèmica. Per tant, inclouen aquelles molècules que inhibeixen processos fisiopatològics com l’entrada de calci a les cèl·lules, l’activació dels radicals lliures o la mort neuronal. Un gran número de fàrmacs neuroprotectors han demostrat efectivitat en models animals reduint considerablement el volum de l’infart i millorant l’evolució funcional però cap d’ells ha demostrat eficàcia en assaigs clínics multicèntrics (25). El grup de treball STAIR (*Stroke Treatment Academic Industry Roundtable*) va plantejar les possibles causes i solucions d’aquest problema que es recullen en unes guies de treball d’utilització en estudis preclínics amb models animals. Les recomanacions inclouen entre d’altres la necessitat de realitzar estudis randomitzats i cecs, utilitzar finestres terapèutiques més llargues, controlar els efectes adversos o utilitzar animals amb comorbiditats i d’espècies diferents (26,27).

El fracàs de la translació a la pràctica clínica de la neuroprotecció ha dut a un gran nombre d’investigadors i empreses del sector bio-farmacèutic a buscar noves estratègies per al tractament de l’ictus com és la neuroreparació.

1.2.3 La neuroreparació

Una altra estratègia terapèutica per al tractament de l'accident cerebrovascular és potenciar la recuperació funcional espontània. Anomenem neuroreparació al conjunt de processos que es produeixen de forma espontània, que es donen més tardanament en la isquèmia cerebral i que, des de les zones del peri-infart, intenten regenerar el teixit danyat i restablir els circuits neuronals per recuperar les funcions sensorials i motores perdudes (28) (**Figura 4**). És important destacar que aquests mecanismes s’activen de forma molt primerenca al mateix temps que es produeixen els processos de mort cel·lular.

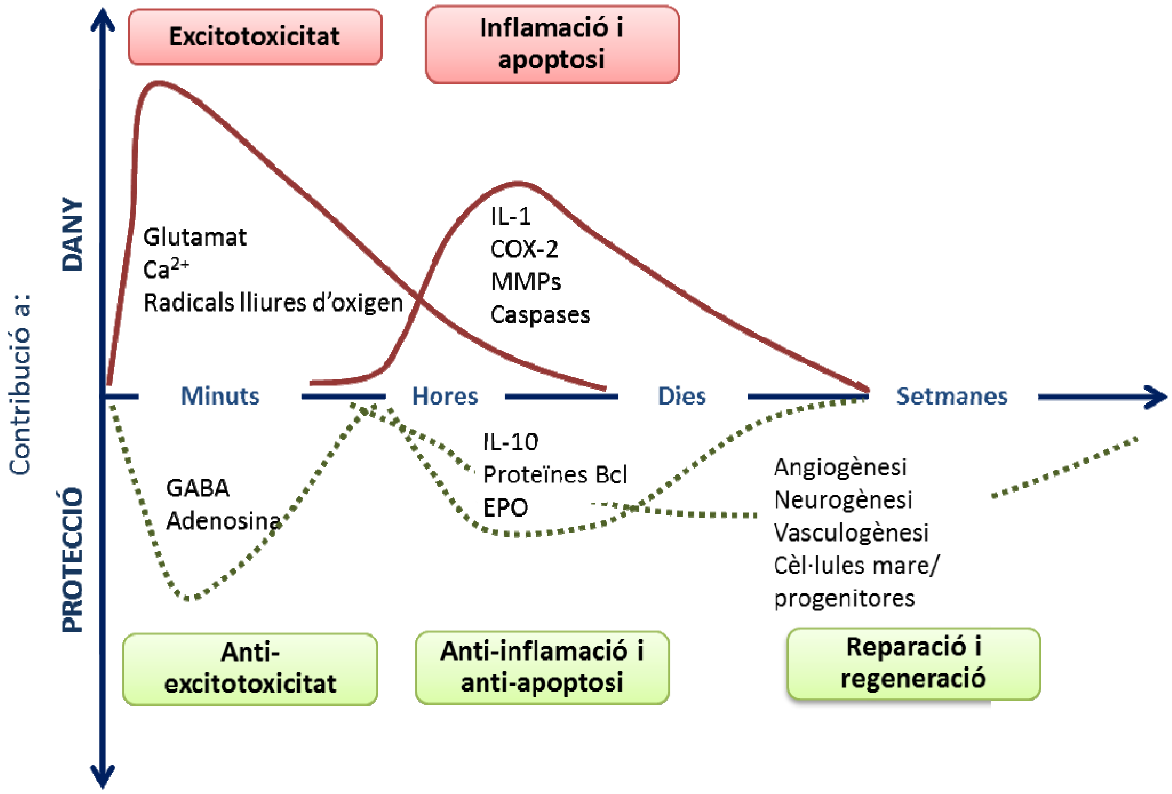

Figura 4. La isquèmia cerebral activa vies complexes de dany cerebral, a les que el teixit cerebral respon estimulant els mecanismes de protecció i reparació (Adaptat de Dirnagl, 2012) (29).

El cervell adult no és tan rígid ni estàtic com es pensava clàssicament. Avui dia sabem que diversos mecanismes de reparació s'activen en resposta a una lesió, incloent l'angiogènesi, la neurogènesi, la sinaptogènesi o l'oligodendrogènesi. El més important és que aquests processos de reparació del cervell poden ser potencialment estimulats mitjançant la rehabilitació, farmacoteràpia o teràpia cel·lular. El principal avantatge en comparació amb la neuroprotecció és la finestra de temps terapèutica, que es pot estendre fins a diverses setmanes o mesos després de la lesió inicial, augmentant així el nombre de pacients que es podrien beneficiar d'aquests tractaments (30). La major part de les teràpies neurorepadores que es troben en fase de desenvolupament pretenen estimular la funció de diferents tipus de cèl·lules del teixit cerebral (incloent cèl·lules mare neurals, cèl·lules endotelials, astròcits, oligodendròcits i neurones), conduint a potenciar la neurogènesi endògena, l'angiogènesi, el creixement axonal i la sinaptogènesi, respectivament, en el teixit cerebral danyat (31). Es creu que potenciar aquests mecanismes de regeneració cel·lular pot contribuir a millorar la funció neurològica després de l'ictus.

1.3 Models animals d'isquèmia cerebral

Com veurem, la major part del coneixement actual de la patologia cerebrovascular es deriva d'estudis experimentals que han ajudat a entendre millor els mecanismes fisiopatològics que contribueixen al desenvolupament d'aquesta malaltia. En el cas de l'ictus la necessitat d'aquests tipus de models és fonamental degut al caràcter agut de la malaltia i per la limitada accessibilitat al cervell humà.

L'objectiu dels models experimentals és generar una situació similar a les condicions observades en la pràctica clínica. Permeten investigar la fisiopatologia d'una malaltia tan complexa com la isquèmia cerebral amb un enfocament reduccionista, que facilita l'estudi dels seus components clínics individuals. Per tant, un model ideal hauria de:

- Reproduir la situació clínica: simular les condicions de la isquèmia cerebral humana i obtenir una lesió al parènquima cerebral similar a l'infart cerebral humà.
- Ser reproduïble.
- Tenir una baixa variabilitat, entre animals i investigadors, sobretot pel que fa a la mida i localització de la lesió.
- Ser senzill de fer i no requerir excessius recursos tecnològics ni tenir un alt cost, per poder ser desenvolupat arreu del món.

D'altra banda, els models animals han de permetre l'avaluació dels danys histològics i l'afectació funcional, així com oferir la possibilitat de realitzar la reperfusió del vas oclòs després de la fase d'isquèmia. En general, els principals problemes de tots els models d'isquèmia cerebral són la seva alta variabilitat entre individus i la seva elevada mortalitat que dificulten la realització d'estudis a llarg termini (32).

Existeixen dos tipus bàsics d'isquèmia cerebral que també classifiquen els models animals: la global, que afecta tot el cervell i la focal, que afecta un sol territori vascular. De cadascuna d'aquestes aproximacions s'han desenvolupat diferents models experimentals que es recullen a la **Taula 4**.

Isquèmia Global	Isquèmia **Completa**	Aturada Cardíaca
		Oclusió aòrtica
		Decapitació
		Torniquet al coll
	Isquèmia **Incompleta**	Hemorràgia o hipotensió
		Isquèmia hipòxica intracranial
		Oclusió de 2 vasos i hipotensió
		Oclusió de 4 vasos
		Oclusió unilateral de l'artèria caròtida comú
Isquèmia Focal	Isquèmia **Focal**	Oclusió de l'artèria cerebral mitja (MCAO), distal o proximal
		MCAO i oclusió ipsilateral de l'artèria caròtida comú
		MCAO i oclusió bilateral de l'artèria caròtida comú
		Infart cerebral espontani
	Isquèmia **multifocal**	Embolització amb coàguls sanguinis
		Embolització amb microesferes
		Foto-trombosi

Taula 4. Principals models d'isquèmia cerebral en rosegadors (Adaptat de Liu i McCullough, 2011)(33). MCAO: oclusió de l'artèria cerebral mitja.

La isquèmia global acostuma a aparèixer després d'una parada cardíaca. Els models d'isquèmia global es basen en la parada cardíaca o en l'oclusió de les grans artèries que irriguen l'encèfal. Existeixen diferents models per a induir la isquèmia global, que produeixen l'afectació de l'hipocamp, el caudoputamen i el neocòrtex. Els més utilitzats són el mètode d'oclusió dels 4 vasos, que consisteix en un oclusió de les artèries caròtides comunes i vertebrals, o el mètode d'oclusió de 2 vasos, on només s'oclouen les caròtides comunes (34). Actualment són poc utilitzats com a models d'ictus.

Els models d'isquèmia focal reprodueixen millor els accidents cerebrovasculars humans, ja que la major part dels ictus isquèmics es deuen a tromboembolismes intracranials (gairebé el 80%). La majoria d'aquests models es centren en l'oclusió de l'artèria cerebral mitja (ACM) per diferents mètodes i aproximacions: l'oclusió mecànica tant a nivell proximal (oclusió de gran vas) com a nivell distal (oclusió de petit vas) per introducció d'un filament, lligament, clip o electrocoagulació, l'oclusió trombòtica ja sigui a través de la injecció de coàguls sanguinis, trombina o endotelina-1 directament a la ACM, o per foto-trombosi local després de la injecció intravenosa de Rosa de Bengala que indueix la formació d'un trombus (34). Es poden classificar en models transitoris o permanents depenent de si es permet o no la reperfusió del teixit isquèmic. El dany isquèmic generat en models d'isquèmia focal depèn del temps de la isquèmia, el lloc on es realitzi l'oclusió, així com d'altres factors com són el sexe, l'espècie i la soca de l'animal, la temperatura corporal o la pressió arterial. En general aquests models són més complexos quirúrgicament que els models d'isquèmia global i la mortalitat és més alta ja que és més fàcil l'aparició de complicacions durant la cirurgia, especialment en els models que afecten els nuclis caudat i putamen (33,35).

L'elecció del model experimental a utilitzar dependrà de l'objectiu de cada estudi i ha de tenir en compte totes les variables esmentades anteriorment. Principalment cal tenir present el procés fisiopatològic que es vol estudiar (per exemple si estem interessats en el dany de la reperfusió, els mecanismes de neuroreparació, aparició de transformacions hemorràgiques, etc.) i la reproductibilitat de les variables de cada model. Caldrà tenir en compte l'àrea cerebral afectada, la recuperació de l'animal i la mortalitat si volem fer estudis a llarg termini o l'existència de reperfusió si volem administrar fàrmacs que arribin a l'àrea infartada (36). En el cas de l'estudi de tractaments neuroprotectors serà necessari que el model desenvolupi aquells processos sobre els que actua la neuroprotecció. Per exemple si estem interessats en la reducció de l'edema o hemorràgia després del tractament trombolític, haurem de triar un model tromboembòlic. D'altra banda, si estem interessats en els processos de reparació ens caldrà un model amb dany tissular que deixi una zona peri-infart molt definida i una baixa mortalitat a llarg termini com podria ser una oclusió distal de l'artèria cerebral mitja (35,37).

1.4 Processos de reparació: neurogènesi i angiogènesi

Els diferents tipus cel·lulars que formen part del parènquima cerebral formen una complexa xarxa d'interaccions, formada per les cèl·lules dels vasos cerebrals (cèl·lules endotelials i perícits), astròcits, neurones i els seus axons i altres cèl·lules de suport, com la micròglia i els oligodendròcits. Aquest complex multicel·lular funcional i estructural s'anomena Unitat Neurovascular (38,39) (**Figura 5**). Les bases empíriques que fonamenten la visió unitària del complex multicel·lular serien que (38) :

a) L'ictus isquèmic és un trastorn vascular amb conseqüències neurològiques.
b) Els tractaments enfocats exclusivament a les neurones han estat poc efectius en neuroprotegir el cervell en assaigs clínics.
c) S'ha demostrat la comunicació entre neurones i cèl·lules endotelials, via astròcits.
d) Les respostes neuronals i vasculars a la isquèmia focal es produeixen simultàniament.

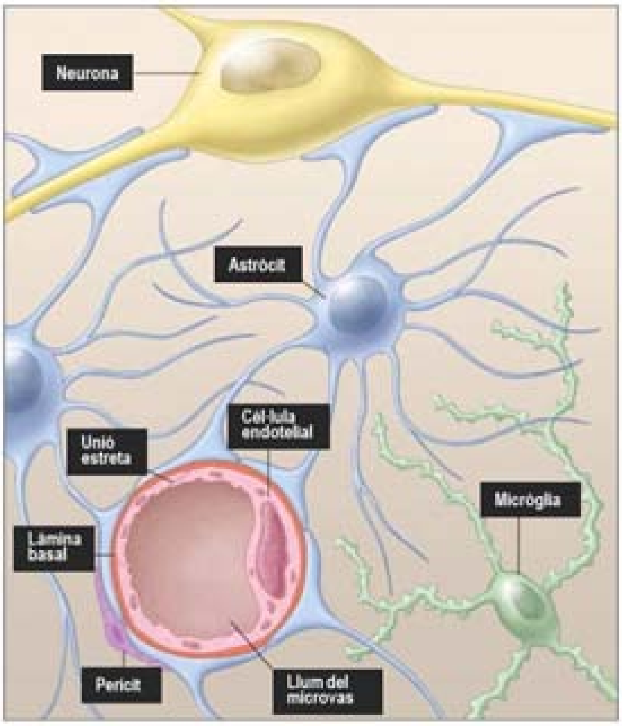

Figura 5. La Unitat Neurovascular. El complex comprèn les neurones, els microvasos en, les cèl·lules de suport i la matriu extracel·lular a través de la qual es produeix la comunicació. Els vasos cerebrals estan formats per l'endoteli (que forma la BHE), la làmina basal i els peus dels astròcits. La comunicació entre les neurones i els microvasos té lloc a través dels astròcits. La micròglia i els perícits també participen en la unitat. (Adaptat de del Zoppo, 2006)(40)

La isquèmia cerebral afecta les cèl·lules que formen la Unitat Neurovascular que veuen alterades les seves funcions fisiològiques, però també desencadena una resposta regenerativa, potenciant els processos de reparació com la neurogènesi i l'angiogènesi que es descriuen a continuació(41).

La **neurogènesi** es defineix com la producció de les cèl·lules del sistema nerviós, per tant les neurones i la micròglia, a partir de les cèl·lules mare i progenitores neurals. En aquest sentit, un dels avenços més importants de finals del segle XX en el camp de la neurociència va ser la constatació d'àrees de neurogènesi en els cervells dels mamífers adults, localitzades en la zona subventricular i la zona subgranular (en la circumvolució dentada de l'hipocamp) de l'encèfal (42). En condicions normals des d'aquestes àrees els neuroblasts migren cap als bulbs olfactoris i l'hipocamp. En estudis experimentals, s'ha demostrat que després de la isquèmia focal es produeix un increment de la neurogènesi en la zona subventricular de l'hemisferi ipsilateral (l'hemisferi afectat per la isquèmia) i també una migració dels neuroblasts des d'aquesta àrea cap a la perifèria de l'infart. En aquesta zona els neuroblasts adquireixen fenotip de neurones madures (43,44). Aquesta activació de la neurogènesi en la zona subventricular després de la isquèmia també té lloc en els humans adults (45,46).

D'altre banda, en la reparació del cervell amb dany isquèmic, conjuntament amb la neurogènesi i els processos de plasticitat neural, també hi tenen lloc processos de plasticitat i remodelació vascular en que hi participen l'angiogènesi i la vasculogènesi (47).

L'**angiogènesi** és el procés mitjançant el qual es produeixen nous vasos sanguinis a partir d'altres ja existents. És un procés àmpliament regulat que es produeix tant en condicions fisiològiques com patològiques. L'angiogènesi té lloc principalment en dues fases: activació i maduració. En la primera es produeix un increment de la permeabilitat vascular, una remodelació de la matriu extracel·lular i una proliferació i migració de les cèl·lules endotelials. Durant la fase de maduració s'inhibeix la proliferació de les cèl·lules endotelials i té lloc la reconstrucció de la làmina basal i el reclutament de perícits i cèl·lules musculars llises per crear l'estructura del vas sanguini i poder restablir el flux sanguini (48,49).

Un fenomen similar però d'origen diferent és la **vasculogènesi**, el procés de formació de vasos sanguinis *de novo*. En l'embrió aquest procés suposa la creació de nous vasos sanguinis a partir dels precursors endotelials, però la identificació de les cèl·lules progenitores endotelials (EPCs, de l'anglès *Endothelial Progenitor Cells*) en l'adult va demostrar l'existència de la vasculogènesi en l'adult més enllà de la fase embrionària (50,51). Les EPCs també contribueixen al desenvolupament dels vasos sanguinis col·laterals mitjançant l'increment del calibre i mida de les connexions arteriolars, procés conegut com arteriogènesi (52).

Estudis en rosegadors amb models d'oclusió permanent o transitòria de l'artèria cerebral mitja (ACM) han demostrat que les cèl·lules endotelials del peri-infart comencen a proliferar a les 12-24h posteriors a la oclusió i que aproximadament entre 2 i 28 dies després de la isquèmia cerebral ja trobem nous vasos en aquesta regió (53,54). En els humans, l'angiogènesi s'inicia en l'àrea del peri-infart del cervell isquèmic 3-4 dies després de l'ictus (55). Resultats obtinguts en models experimentals, mostren que la potenciació de l'angiogènesi molt aviat després de la isquèmia amb l'administració de factors de creixement com el factor de creixement de l'endoteli vascular (VEGF), provoca un increment de la permeabilitat vascular i el trencament de la BHE (56). En canvi el tractament més tardà augmenta l'angiogènesi en el peri-infart i millora la recuperació neurològica (56-58). D'altres estudis demostren un increment en l'expressió de factors inhibidors de l'angiogènesi com l'endostatina i la trombospondina-1 i -2 (TSP-1 i TSP-2) durant la fase aguda de l'ictus (59-61). Les TSP-1 i -2, d'altra banda, estan relacionades amb la plasticitat sinàptica i la seva deficiència provoca un empitjorament en la recuperació funcional dels animals isquèmics, sense afectar per això l'angiogènesi (62). Per tant, els estudis publicats fins al moment mostren que es produeix una activació tant dels promotors com dels inhibidors de l'angiogènesi després de la isquèmia cerebral.

Després de l'ictus i del dany cerebral, els processos angiogènics i neurogènics derivats estan estretament relacionats (63). El "nínxol neurovascular" defineix aquests complexos mecanismes de senyalització cèl·lula-cèl·lula entre els precursors neuronals de les zones subventricular i subgranular i l'endoteli cerebral, que també es mantenen després de l'isquèmia. Els neuroblasts provinents de la zona subventricular migren estretament associats als vasos sanguinis cap a la perifèria de l’infart on té lloc l’angiogènesi (47). En models animals s’ha demostrat que en aquestes àrees s’expressen factors de creixement com el factor derivat de cèl·lules estromals 1 (SDF-1) o l’angiopoietina 1 (Ang-1) que actuen directament sobre receptors neurals (CXCR4 i Tie2, respectivament), provocant la migració dels neuroblasts a les zones perifèriques a l’infart. A més, la inhibició farmacològica de l’angiogènesi amb endostatina redueix dràsticament la migració dels neuroblasts cap al peri-infart (64,65).

1.5 Teràpies per a la potenciació de l'angiogènesi en l'ictus

D'entre els processos de reparació que hem comentat l'angiogènesi té un paper central en la regeneració tissular. De manera directa permet l'arribada de la sang, i per tant oxigen i nutrients, al teixit afectat però, a més, també actua de forma indirecta proporcionant una xarxa de suport per a la migració de cèl·lules progenitores i enriquint la matriu extracel·lular amb factors de creixement a través de la seva secreció per les noves cèl·lules endotelials (48).

1.5.1 Teràpies basades en cèl·lules

Després d'un ictus la pèrdua cel·lular és molt elevada. Això suposa un repte per a les teràpies cel·lulars en comparació amb d'altres malalties on aquesta pèrdua pugui ser menor o més restringida en quant als tipus cel·lulars afectats. No només les neurones, sinó també les cèl·lules glials de suport i els vasos sanguinis necessiten ser reparats o la seva funció substituïda. A més, l'edema pot limitar l'empelt de les noves cèl·lules, mentre que l'ambient tissular hostil (estrès oxidatiu, inflamació, etc)pot posar-ne en perill la supervivència en els primers moments després de l'ictus. Així doncs, l'aproximació més raonable sembla ser el trasplantament de les cèl·lules en moments més posteriors passada la fase hiperaguda de la malaltia, evitant la neurodegeneració secundària i potenciant els mecanismes de reparació cerebrals (31,66).

Les cèl·lules mare existents que han demostrat un potencial reparador desprès de la isquèmia cerebral tenen una gran diversitat d'orígens i funcions (**Taula 1**) i el seu potencial terapèutic s'ha estudiat en models experimentals com a promotores de l'angiogènesi (67). Els nous vasos i capil·lars que es formen, a banda del propi benefici de millorar la perfusió del teixit, poden produir factors que estimulen la remodelació del teixit cerebral isquèmic de manera directa sobre les cèl·lules endotelials madures o indirecta a través de l'estimulació de la funció d'altres tipus cel·lulars (68-70). Per exemple, l'expressió de BNDF (factor neurotròfic derivat del cervell), VEGF, VEGFR2 i metal·loproteïnases de matriu (MMPs) s'ha associat a la remodelació del teixit cerebral danyat per la isquèmia, la formació de noves sinapsis i l'atracció dels neuroblasts en un model experimental en rosegadors (71).

Taula 1. Breu resum dels tipus de cèl·lules mare (Adaptat de Gutiérrez-Fernández et al. 2012)(72).

Abreviatura i nom en anglès	**Nom**	**Descripció**	**Estudis en models animals d'isquèmia cerebral**
ESC, E*mbryonic Stem Cells*	Cèl·lules mare embrionàries	Cèl·lules mare pluripotents i amb capacitat d'autorenovació derivades de la massa cel·lular interna del blastocist	Wei et al., 2005(73); Nagai et al., 2010(74)
IPS, *Inducible Pluripotent Stem cells*	Cèl·lules mare pluripotents induïdes	Cèl·lules somàtiques de l'adult derivades de teixits normals modificades mitjançant enginyeria genètica, que s'assemblen a les cèl·lules mare pluripotents i tenen potencial d'autorenovació i de diferenciació a diferents tipus cel·lulars.	Jiang et al., 2011(75); Yuan et al., 2013(76)
NSC, *Neural Stem cell*	Cèl·lules mare neurals	Cèl·lules amb potencial d'autorenovació capaces de diferenciar-se en els tipus cel·lulars cerebrals neurals més rellevants (neurones, astròcits i oligodendròcits)	Andres et al., 2011(77); Takahashi et al., 2008(78)
BMSCs, *Bone Marrow Stem Cells*		Cèl·lules mare derivades de medul·la òssia	
HSCs, *Hematopoietic Stem Cells (CD34+)*	Cèl·lules mare hemopoètiques	Poblacions heterogènies de cèl·lules multipotents capaces de diferenciar-se en tots els tipus de cèl·lules sanguínies (tant mieloide com limfoide)	Keimpema et al., 2009(79); Nystedt at al., 2006(80); Taguchi et al., 2004(81)
EPCs, *Endothelial Progenitor Cells (CD34+)*	Cèl·lules progenitores endotelials	Cèl·lules sanguínies circulants capaces de diferenciar-se en cèl·lules endotelials i amb funcions angio-vasculogèniques.	Fan et al., 2010(82); Moubarik et al., 2011(83)
MSC, *Bone Marrow Mesenchymal Stem Cells (CD34-)*	Cèl·lules mare mesenquimals derivades de medul·la òssia	Cèl·lules mare multipotents circulants en sang amb capacitat de diferenciació en diferents teixits i de reparació dels teixits danyat	Li et al., 2001 (84); Gutierrez-Fernandez et al., 2011(85); (72){{145 Gutierrez-Fernandez,M. 2012}}(65) Yang et al., 2010 (86)

Algunes evidències experimentals van mostrar en un model murí d'oclusió de l'artèria cerebral mitja que el reclutament de cèl·lules procedents de medul·la òssia induïa la revascularització després de la lesió isquèmica i augmentava l'estabilització dels vasos sanguinis (87). A més, l'administració sistèmica de cèl·lules CD34+, derivades de sang del cordó umbilical humà, a ratolins immunocompromesos i sotmesos a 48h d'isquèmia cerebral, estimulava l'angiogènesi potenciant la neovascularització al voltant del còrtex afectat (81). Les EPCs, que descriurem en profunditat més endavant, són un dels tipus cel·lulars compresos en la població de cèl·lules CD34+. Aquestes cèl·lules també han demostrat recentment la seva efectivitat potenciant l'angiogènesi, reduint el volum de l'infart i millorant el dèficit neurològic en models d'isquèmia cerebral en rata i ratolí (82,83).

Un altre dels tipus cel·lulars estudiats han estat les cèl·lules mare neurals. La seva administració ha demostrat potenciar la neurogènesi, la gliogènesi i l'activitat dendrítica en el cervell isquèmic (77). Les cèl·lules mare neurals també estan indirectament relacionades amb l'angiogènesi. En aquest sentit s'ha descrit que el trasplantament de cèl·lules mononuclears de la medul·la òssia, després de la isquèmia, activa la proliferació de les cèl·lules mare neurals endògenes, i aquest procés s'acompanya amb un increment de l'angiogènesi (88).

Més recentment, s'ha començat a treballar amb les cèl·lules mare pluripotents induïdes (iPS). Les iPS són cèl·lules madures que són reprogramades per esdevenir pluripotents, i per tant capaces de desenvolupar-se en tots els teixits del cos. En provenir de l'adult, les iPS són cèl·lules mare lliures de conflictes ètics i també s'han estudiat en la isquèmia cerebral. Després de la seva administració en l'animal isquèmic, les cèl·lules es diferencien en neurones i astròcits i produeixen una millora funcional en els animals tractats (75,76). Malgrat els resultats prometedors, cal anar en compte amb les cèl·lules mare pluripotents induïdes, ja que alguns estudis han mostrat que les cèl·lules no diferenciades poden desenvolupar tumors en determinades condicions (89,90). Finalment, una de les cèl·lules més estudiades són les cèl·lules mare mesenquimals derivades de la medul·la òssia. Avui dia ja s'ha demostrat que l'administració de les cèl·lules mare mesenquimals en animals isquèmics redueix el dèficit neurològic, potencia l'angiogènesi, i són capaces de diferenciar-se en astròcits i neurones (84,86).

Així doncs, totes aquestes cèl·lules constitueixen teràpies prometedores per l'ictus, de fet, algunes poblacions cel·lulars CD34+, cèl·lules mare mesenquimals o EPCs es troben ja en les primeres fases d'assaigs clínics en humans (91); www.clinicaltrials.gov.

1.5.2 Factors promotors de l'angiogènesi

Una alternativa a la teràpia cel·lular són les teràpies basades en l'administració directa de factors pro-angiogènics que s'expressen de forma endògena en el teixit nerviós i que són secretats per diferents tipus cel·lulars. Aquestes teràpies tenen l'avantatge d'evitar les possible reaccions immunològiques a un trasplantament cel·lular, així com evitar la possible limitació en el nombre de cèl·lules que es poden administrar.

Un dels factors promotors de l'angiogènesi més estudiat és el VEGF. L'administració directa de VEGF en models murins d'isquèmia cerebral s'ha utilitzat com a neuroprotector, reduint el volum de l'infart, i com a agent neuroreparador estimulant l'angiogènesi (58,92,93). Malgrat els nombrosos estudis mostrant resultats prometedors del VEGF també s'ha evidenciat que el seu efecte sobre la permeabilitat vascular pot conduir a l'obertura BHE, incrementar l'edema cerebral o la vasodilatació (94). D'altra banda, la transferència del gen del factor de creixement dels hepatòcits (HGF) en un model en rata, va demostrar una reducció del volum d'infart i va potenciar l'angiogènesi a nivell local sense afectar l'estabilitat de la BHE (95). L'administració després de la isquèmia d'altres factors pro-angiogènics com la leptina o el factor de creixement de fibroblasts-2 (FGF-2) també ha demostrat produir un increment de l'angiogènesi en models murins (96,97). A més, l'administració intracerebral de SDF-1 en rates isquèmiques va disminuir el volum de l'infart, s'incrementà la densitat vascular i va produir una atracció de cèl·lules provinent de la medul·la òssia al teixit danyat (98).

D'altres factors de creixement, incloent el G-CSF i l'eritropoetina (EPO), han estat estudiats també pel seu potencial neuroreparador però la limitació de la dosi, degut a la seva toxicitat, han frenat el seu desenvolupament com a teràpies per l'ictus (99). En aquest sentit, un assaig clínic multicèntric de 328 pacients amb ictus tractats amb G-

CSF o placebo no va aconseguir mostrar un benefici en el tractament als 90 dies (100). D'altra banda l'EPO ha demostrat estimular l'angiogènesi, la neurogènesi i la recuperació funcional després de la isquèmia en rates sotmeses a isquèmia cerebral relacionat amb un augment de l'expressió de VEGF i del BDNF al cervell (101). Els assaigs clínics amb EPO es van aturar en produir-se un augment de les hemorràgies i la mortalitat en els pacients d'ictus que van rebre la combinació d'EPO i rt-PA (102).

Una aproximació terapèutica interessant és la combinació de diferents factors de creixement. Per exemple, la combinació de VEGF i Ang-1 va demostrar ser més efectiva en la reducció del volum de la lesió així com en l'estimulació de l'angiogènesi, en ratolins isquèmics, que el tractament amb només VEGF (103). En aquest sentit, diferents estudis semblen indicar que l'efecte paracrí de les cèl·lules mare i progenitores té un rol fonamental en els processos de reparació tissular i que el tractament amb el medi condicionat obtingut d'aquestes cèl·lules, ric en els factors de creixement secretats seria una estratègia terapèutica prometedora (104,105). L'ús del medi condicionat de cèl·lules mare o progenitores com a teràpia regenerativa ja ha donat resultat positius en estudis experimentals en d'altres patologies. Per exemple, el tractament amb medi condicionat obtingut de cèl·lules mare mesenquimals o de cèl·lules mare derivades de teixit adipós van millorar la cicatrització de ferides de la pell (106). A més, el medi condicionat d'EPCs va demostrar en un model d'isquèmia perifèrica en rata estimular la neovascularització, mostrant resultant tant efectius com la teràpia cel·lular (107). En l'ictus no és un camp tan explorat, però recentment també s'han reportat resultats prometedors. La infusió de medi condicionat de cèl·lules mare humanes derivades de teixit adipós en rates sotmeses a un model d'isquèmia cerebral va induir una recuperació funcional i estructural, com a conseqüència d'una major neovascularització, una reducció de l'apoptosi de les cèl·lules neurals i una menor astrogliosi (108).

1.6 Les cèl·lules progenitores endotelials (EPCs)

1.6.1 Definició

Les EPCs són cèl·lules mare derivades del moll de l'os i d'origen hematopoètic presents en individus adults, amb capacitat per diferenciar-se a cèl·lula endotelial madura i de formar nous vasos sanguinis totalment funcionals (51). El descobriment per Asahara d'aquestes EPCs en sang perifèrica en humans el 1997 va canviar completament la nostra comprensió sobre la formació de nous vasos sanguinis després del dany vascular en l'adult. Fins aleshores es creia que els processos d'**angiogènesi** (producció de nous vasos sanguinis a partir d'altres ja existents) i arteriogènesi eren els únics responsables de la neovascularització en l'individu adult, i en canvi, la **vasculogènesi** (procés de formació de vasos sanguinis *de novo*) es considerava que es donava només durant la fase embrionària. Asahara i els seus col·laboradors van demostrar per primer cop que un subtipus de cèl·lules mononuclears sanguínies positives per CD34 i VEGFR-2 i cultivades sobre fibronectina tenien la capacitat de formar estructures similars a tubs *in vitro* i podien incorporar-se als capil·lars de les regions isquèmiques en un model animal d'isquèmia perifèrica (51). Aquestes cèl·lules són les anomenades EPCs, que després d'una lesió tissular es mobilitzen des de la medul·la òssia cap al torrent sanguini i poden migrar fins al lloc de la lesió vascular (on es diferencien en cèl·lules endotelials) per induir la neovascularització o reparació endotelial.

Es creu que les EPCs posseeixen característiques funcionals i estructurals de cèl·lules mare (com la capacitat clonogènica), així com de cèl·lules endotelials madures (capacitat de formar estructures vasculars). Urbich i Dimmeler han definit posteriorment les EPCs com a cèl·lules no endotelials que mostren característiques i capacitat clonogènica de cèl·lules mare i que posseeixen la capacitat de diferenciar-se a cèl·lules endotelials (109). A dia d'avui no s'ha identificat un únic marcador que identifiqui aquestes cèl·lules així que la definició fenotípica majoritàriament acceptada és la coexpressió dels marcadors de superfície CD34 i VEGFR-2 (també anomenat receptor del domini d'inserció de quinasa, KDR) que pot incloure el marcador CD133 (110). El CD133 incrementaria l'especificitat ja que no és expressat per les cèl·lules endotelials madures al contrari del CD34, que podria trobar-se en algunes d'elles, però també hi ha estudis que han mostrat que el CD133 definiria una població d'EPCs més immadura i que no sempre esdevindrien cèl·lules endotelials madures (111). Hi ha altres marcadors de superfície que han estat descrits per definir subpoblacions d'EPC com el factor de von Willebrand (vWF), CD31 (molècula d'adhesió cel·lular endotelial de plaquetes), CD62E (E-selectina), CD144 (VE-cadherina) i el CXCR-4 (112). La coexpressió d'aquests marcadors caracteritza un subtipus específic de cèl·lules progenitores en una etapa de maduració específica, per tant la seva expressió no és sempre estable. Per exemple, l'expressió de CD34, el primer marcador descrit per definir les EPCs, disminueix amb el temps a mesura que es diferencien cap a cèl·lules endotelials (51). L'heterogeneïtat d'aquesta població fa que encara no tinguem un únic marcador per definir-la, i que per tant, la combinació de marcadors hematopoètics, endotelials i progenitors hagi estat i segueixi sent l'estratègia més utilitzada tot i que sense un consens a dia d'avui.

Actualment, està creixent la idea que hi ha diferents tipus de cèl·lules circulants en sang que participen en el procés de formació de nous vasos sanguinis i la reparació vascular. Aquest fet genera controvèrsia sobre si qualsevol cèl·lula que participa en processos de neovascularització i mostra característiques de llinatge hemopoètic ha de ser considerada una veritable EPC (en anglès *true EPC*), o en canvi, es reserva el terme EPC per les cèl·lules progenitores de llinatge endotelial. Yoder proposa que una EPC s'hauria de definir amb les següents propietats fonamentals (113):

- Una cèl·lula circulant que dóna lloc a una progènie amb potencial prolífic clonal i una diferenciació restringida al llinatge endotelial.
- Capacitat per formar tubs similars a capil·lars *in vitro.*
- Capacitat de formar vasos sanguinis estables que es converteixin en una part integrada del sistema circulatori d'acollida quan s'implanten en teixits.
- Capacitat per formar i remodelar la capa íntima de les estructures arterials, venoses i capil·lars.

Trobar un marcador de superfície cel·lular únic que permeti la identificació i l'aïllament de les cèl·lules que presenten aquestes propietats permetrà aclarir sense dubtes la identitat de les EPCs i definir millor les diferents subpoblacions amb les que es treballa en la actualitat.

1.6.2 Cultiu de les EPCs

A partir dels marcadors de superfície descrits anteriorment les EPCs es poden aïllar de la resta de cèl·lules mononuclears per citometria de flux. En el cas dels cultius cel·lulars, distingim les EPCs perquè són les cèl·lules que s'adheriran a la base de fibronectina tal com va definir inicialment Asahara i que creixeran en medis específics per a la supervivència de cèl·lules endotelials (51).

A partir de les seves característiques en cultiu en fibronectina les EPCs es poden dividir en dos subtipus que comprenen dues poblacions cel·lulars diferents morfològicament i funcionalment: les *early-EPCs*, també anomenades *circulating angiogenic cells* (CACs i les *late-EPCs*, també conegudes com *late outgrowth endothelial cells* (OECs) o *endothelial colony-forming cells* (ECFCs). Les *early-EPC* apareixen més aviat en el cultiu, als 5-7 dies, i tenen una morfologia fusiforme (en anglès *spindle-shape*), mentre que les OECs apareixen a partir de les dues setmanes de cultiu formant una monocapa cel·lular clonogènica de tipus llamborda (en anglès *cobblestone*)(114) (**Figura 6**). A més dels cultius en fibronectina, en la actualitat diversos autors descriuen l'aïllament d'EPCs en bases de col·lagen, on després d'uns 14 dies de cultiu de les cèl·lules mononuclears de la sang s'obtenen ECFCs (111,115).

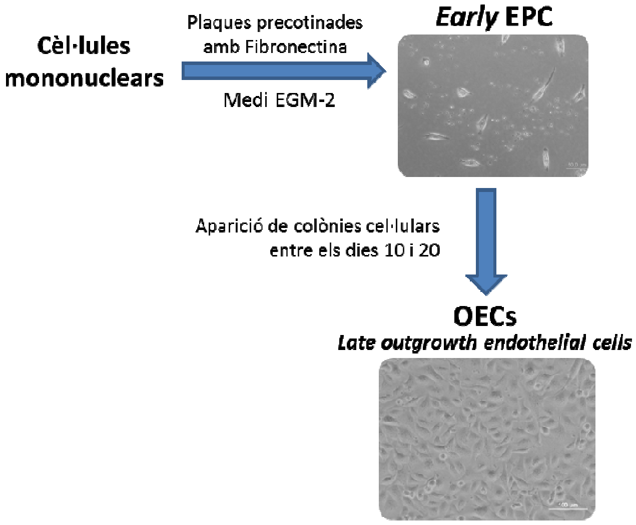

Figura 6. Mètode de cultiu de les EPCs: Les cèl·lules mononuclears són aïllades de la sang perifèrica o de la melsa (en ratolins) per gradient de densitat amb Ficoll i sembrades en plaques precotinades amb fibronectina. Les primeres cèl·lules que apareixen en cultiu són les anomenades *early* EPCs o CACs. Posteriorment, en alguns cultius, apareixen colònies de OECs amb capacitat clonogènica.

A més, les OECs són les úniques amb potencial per formar estructures vasculars *de novo* (tubulogènesi in vitro). Tot i que les *early*-EPCs no tenen capacitat tubulogènica, secreten factors de creixement que estimulen aquesta capacitat i poden incorporar-se a estructures vasculars existents (**Taula 2**) (114,116,117).

Taula 2. Característiques principals de les dues poblacions d'EPCs identificades. (Adaptat de Yoon et al., 2005 i Navarro et al., 2007)(117,118)

	***Early* EPC**	**OECs**
Creixement *in vitro*	Aparició primerenca	Creixement tardà
Marcadors de superfície	CD34, VEGFR-2, CD14, CD133, Dil-actLDL, UEA1-lectin	vWF, CD31, VEGFR-2, Dil-actLDL, UEA1-lectin
Formació de túbuls	No	Sí
Capacitat clonogènica	Baixa	Alta
Secreció de citocines (VEGF, IL-8, HGF, G-CSF)	Nivells alts	Nivells baixos
Producció d'Òxid Nítric	Nivells baixos	Nivells alts
Tipus de població	Heterogènia	Homogènia
Potencial angio-vasculogènic	Bo	Bo

Dil-actLDL: acetylated low-density lipoprotein; IL-8: interleuquina-8; UEA1-lectin: ulex europaues aglutinin 1-lectin.

1.6.3 Reclutament i mobilització de les EPCs

L'alliberament de les EPCs des de la medul·la òssia està regulat per l'acció d'una varietat de factors de creixement, enzims, lligants i receptors de la superfície. Alguns dels factors que activen la mobilització de les EPCs en resposta a una isquèmia perifèrica o un ictus isquèmic són el factor induïble per hipòxia 1α (HIF-1α), el VEGF, el SDF-1 o l'EPO (110).

D'altres autors han descrit que dins la medul·la òssia diferents proteases com les elastases, la catepsina G i la metal·loproteïnasa de matriu 9 (MMP-9) trenquen els enllaços entre les EPCs i les cèl·lules estromals provocant l'alliberació i la migració de les EPCs a la circulació perifèrica (119,120). Posteriorment, les EPCs migren cap al teixit afectat, com pot ser l’àrea del peri-infart, atretes per gradients de citocines. Allà actuen de forma paracrina estimulant la proliferació de cèl·lules endotelials i la seva estabilització o es diferencien en cèl·lules endotelials madures participant en la formació de nous vasos (120,121).

Fins ara, no s’ha identificat de forma clara quan una cèl·lula progenitora endotelial es pot considerar una cèl·lula endotelial madura totalment diferenciada. Una possibilitat podria ser la pèrdua de l’expressió de CD133 i una expressió paral·lela o subsegüent del factor de von Willebrand conjuntament amb l'aparició d'altres característiques endotelials. S’especula si el punt de partida d'aquest procés de diferenciació podria ser la migració de les EPCs des de la medul·la òssia a la circulació sistèmica. El procés es completaria després de l’adhesió i inserció en la monocapa de cèl·lules endotelials madures (122).

1.6.4 Les EPCs com a marcador de risc en l’ictus

A banda de com a teràpia promotora de l’angiogènesi després d’un ictus isquèmic, les EPCs (en diferents subtipus) també han estat estudiades també com a possible biomarcador en les malalties cardiovasculars ja que els seus nivells i funció podrien ser marcadors de la funció vascular o del risc de patir algun esdeveniment cardiovascular. Per aquest motiu han estat analitzades en diversos estudis observacionals en pacients d’ictus isquèmic, en algun estudi també s’inclouen malalts d’AIT, per avaluar el seu impacte com a factor diagnòstic pronòstic (**Taula 3**).

Taula 3. Estudis observacionals que analitzen el paper de les EPCs després de l'ictus isquèmic.

Estudi	Població	Mètode d'estudi/ Marcadors EPCs	Resultats
Taguchi et al., 2004(123)	Pacients d'ictus	Citometria de flux CD34, CD133	Correlació positiva de cèl·lules CD34+ amb flux sanguini cerebral, més CD133+ en pacients amb menys nº d'infarts
Ghani et al., 2005(124)	Pacients d'ictus (agut i crònic)	Cultiu (recompte de CFU) CD31, vWF	Nivells inferiors en pacients d'ictus (agut i crònic) vs controls
Sobrino et al., 2007(125)	Pacients d'ictus	Cultiu (recompte de CFU)	Major nombre de CFUs correlaciona amb bon pronòstic funcional
Yip et al., 2008(126)	Pacients d'ictus i controls	Citometria de flux CD31, CD34, VEGFR-2, CD62	Nivells alts d'EPCs associats amb bon pronòstic neurològic, nivells més alts en pacients que en controls
Chu et al., 2008(127)	Pacients d'ictus (agut i crònic) i controls	Cultiu (recompte de CFU) i assaig funcional (efecte en cèl·lules endotelials de sobrenedants de CFU)	Menor nº de CFU i reducció de la capacitat tubulogènica en pacients d'ictus agut respecte ictus crònic o controls
Cesari et al., 2009(128)	Pacients d'ictus	Citometria de flux Recompte de cèl·lules progenitores circulants CPC (CD34,CD133) i EPC (CD34, CD133, VEGFR-2)	Bon pronòstic funcional associat a major nº de CPC però no amb EPCs
Bogoslovsky et al., 2010(129)	Pacients d'ictus	Citometria de flux CD34, CD133, VEGFR-2	Nivells més alts EPC eren indicatius de volums més petits de lesió aguda, lesió final, i creixement de la lesió
Navarro-Sobrino et al., 2010(130)	Pacients d'ictus (agut i subagut) i controls	Citometria de flux (CD34, CD133, VEGFR-2) i cultiu (recompte i assaig tubulogènesi)	Major nº d'EPCs en pacients d'ictus agut que en controls. Correlació positiva nº EPCs i la severitat inicial de l'infart. EPCs de pacients d'ictus subagut mostren major funcionalitat *in vitro.*
Massot et al., 2013(131)	Pacients d'ictus o AIT amb aterosclerosi intracranial i controls	Citometria de flux (CD31, VEGFR2	No diferències en el nombre d'EPCs entre pacients amb aterosclerosi intracranial i controls. Més nombre d'EPCs en pacients amb major extensió de la aterosclerosi intracranial.
Paczkowska et al., 2013(132)	Pacients d'ictus isquèmic i hemorràgic i controls	Citometria de flux per *early* (CD34, VEGFR-2; CD133) i *late* EPC (CD34, VEGFR-2)	Major nº d'*early* i *late* EPCs en pacients d'ictus que en controls

CFU: unitats formadores de colònies.

Els resultats dels estudis són divergents en alguns casos. Per exemple pel que fa als nivells d'EPCs, en estudis realitzats en pacients i controls sans alguns autors mostren una disminució en pacients amb ictus mentre que d'altres n'han descrit un augment respecte als controls. Les divergències en la metodologia emprada en els diferents treballs així com la heterogeneïtat tant dels pacients com dels controls inclosos dificulta la interpretació dels resultats i fa necessari estudis més amplis on s'estudiïn les diferents subpoblacions conegudes, on s'analitzin els nivells d'EPCs per diferents tècniques, i on es determini la capacitat funcional d'aquestes cèl·lules després de l'ictus isquèmic així com establir un rang de valors estàndard per als controls sans. D'altra banda, algun dels mètodes d'estudi utilitzats en aquests estudis, com el recompte d'unitats formadores de colònies (CFUs) actualment ja no s'utilitza, ja que es va veure que es tractava de cèl·lules més relacionades amb poblacions de monòcits o macròfags (113).

1.7 Les Metal·loproteïnases de Matriu (MMPs)

1.7.1 Definició de les MMPs

Les metal·loproteïnases de matriu comprenen una família de proteases dependents de zinc implicades en la regulació de la matriu cel·lular i amb capacitat per a degradar diferents components d'aquesta i de processar diverses molècules bioactives en ella. S'han descrit més de 25 MMPs secretades o de membrana diferents. Clàssicament s'han classificat segons el substrat que preferentment degraden en col·lagenases (MMP-1, -8 i -13), gelatinases (MMP-2 i -9), estremolisines (MMP-3, -10 i -11), metal·loelastases (MMP-12) i matrisilines (MMP-7, -26). Tot i que posteriorment es va adoptar una nova classificació segons les seves característiques funcionals degut a la descripció de nous substrats, la classificació clàssica segueix sent la més utilitzada (133). Encara que es troben codificades en diferents gens, aquestes endopeptidases comparteixen elements estructurals (com un propèptid amino-terminal, un domini catalític i un domini hemopexina) i funcions (134). Totes les MMPs es produeixen en la seva forma latent i esdevenen proteïnes secretades o transmembrana. Les MMPs són sintetitzades i secretades a l'espai extracel·lular com zimògens inactius, amb un domini propèptid. El trencament de la unió entre aquest domini i el domini catalític produeix l'activació de les MMPs. Aquesta activació es pot produir per diferents mecanismes tot i que es considera que el principal és per la proteòlisi directa per altres proteases o MMPs. Algunes de les proteases activadores són la plasmina, el t-PA i l'activador del plasminogen tipus uroquinasa (uPA) (135). L'activitat catalítica de les MMP està regulada a múltiples nivells, incloent la transcripció, la secreció, l'activació i la inhibició post-transcripcional. La inhibició està principalment regulada per la família dels inhibidors tissulars de metal·loproteïnases (TIMP) que en els mamífers compta amb quatre proteïnes: TIMP-1, TIMP-2, TIMP-3 i TIMP-4 (136) però també per molt fàrmacs sintetitzats (SB-3CT, BB-94, GM6001, Minociclina, etc.) (137).

Sabem que més enllà de la degradació enzimàtica de components de la matriu extracel·lular, d'entre les múltiples funcions de les MMPs es troben l'escissió dels receptors de la superfície cel·lular, l'activació o inactivació de les quimiocines i citocines, i l'alliberament de lligands apoptòtics. També participen en la proliferació, la migració i la diferenciació cel·lular, l'angiogènesi i l'apoptosi en processos fisiològics i patològics (138,139).

1.7.2 Les MMPs en la isquèmia cerebral

Les MMPs juguen un paper fonamental tant a la fase aguda com a la fase crònica de l'ictus. Les MMPs poden contribuir al dany neuronal i vascular, al creixement de l'infart i a l'aparició d'hemorràgies durant la fase hiperaguda de l'ictus, mentre que en fases més tardanes podrien ajudar en els processos de reparació tissular i en la modulació de la plasticitat neurovascular (**Figura 7**).

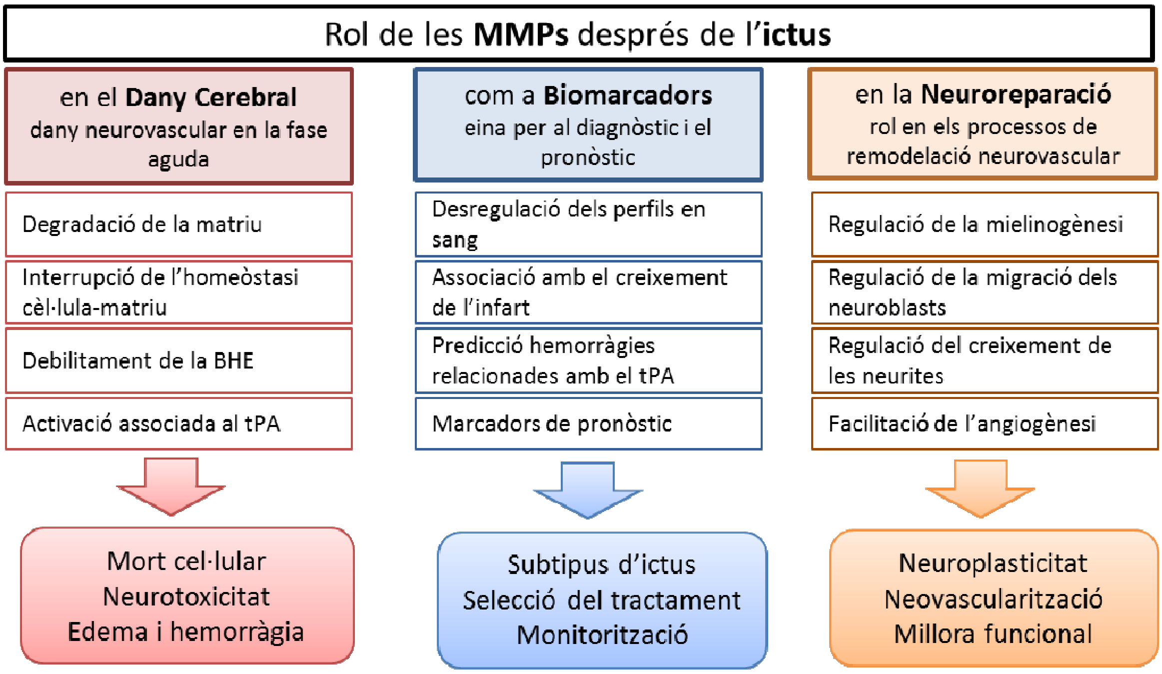

Figura 7. Esquema dels diferents rols que juguen les MMPs després de l'ictus: implicació en el dany cerebral, la neuroreparació i la seva utilitat com a biomarcadors (Adaptat de Rosell et al, 2008).

Durant la fase aguda, les MMPs juguen un paper patològic centrat en degradar substrats de la matriu extracel·lular que són essencials per a la correcta senyalització cel·lular i homeòstasi a la unitat neurovascular. Les MMPs poden degradar components de la làmina basal (col·lagen IV, laminina, etc.), debilitant l'estabilitat dels vasos i per tant, els predisposen a petites fuites de sang i al seu trencament. S'ha descrit que després de la isquèmia cerebral es produeix un augment en l'expressió de diverses MMPs, tant en estudis en mostres humanes com en models experimentals

(140-143). En teixit cerebral humà s'han trobat elevades la MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10 i MMP-13 (144,145), destacant la presència de MMP-9 amb la degradació del col·lagen IV i el trencament de la BHE (142). La infiltració de neutròfils que es produeix després de la isquèmia cerebral es relaciona en models animals amb l'expressió cerebral de MMP-9, demostrant el rol dels neutròfils en l'alliberament de MMP-9 que també s'ha comprovat en cèl·lules humanes (146,147). En models animals d'isquèmia cerebral l'expressió de MMPs també es relaciona amb la disrupció de la BHE, la formació d'edema i l'aparició de transformacions hemorràgiques (139,140). En aquests models s'ha demostrat que el tractament amb inhibidors de les MMPs redueix l'edema i el volum de la lesió com també la inducció de la isquèmia en ratolins deficients per MMP9 (148,149).

D'altra banda, en fases més tardanes de la malaltia, les MMPs són unes proteïnes importants i necessàries per a la remodelació i reparació tissular, i com veurem més endavant participen en els processos d'angiogènesi i neurogènesi. A banda de remodelar la matriu extracel·lular per permetre la mobilització, el creixement i la migració de cèl·lules, les MMPs també participen en el procés angiogènic ajudant en l'alliberació de factors tròfics i pro-angiogènics. Per exemple, poden trencar molècules de la matriu o de la superfície cel·lular com el VEGF i alliberar-lo en la seva forma bioactiva (150). S'ha comprovat que les MMPs també afavoreixen l'angiogènesi trencant les adhesions entre cèl·lules endotelials i permetent la migració endotelial i posterior creixement vascular (151), sent passos necessaris per a la formació de noves estructures tubulars *in vitro* (152). En relació amb la neurogènesi, les MMPs participen en la migració i la diferenciació de les cèl·lules mare i progenitores neurals (153). En el cervell adult les àrees de neurogènesi més importants són la zona subventricular i la zona subgranular de l'hipocamp. Els neuroblasts migren des d'aquestes àrees a la zona afectada, i aquesta migració es potencia per acció de la MMP-2 i -9 secretades per les cèl·lules endotelials (154). A més, després de la isquèmia cerebral es produeix una sobreexpressió de la MMP-9 a la zona subventricular que col·localitzava amb neuroblasts positius per al marcador de proliferació BrdU (155). La MMP-3 és una altra de les MMPs que incrementa la seva expressió després de la isquèmia cerebral, i s'ha trobat en diferents tipus cel·lulars com neurones isquèmiques, oligodendròcits, microvasculatura i macròglia reactiva (156).

En resum hi ha un gran nombre d'estudis que mostren el rol deleteri de les MMPs en la fase aguda de l'ictus però també s'ha descrit la seva implicació en la neuroreparació en la fase crònica de la malaltia. Per tant, s'ha proposat que la inhibició de les MMPs en la fase aguda podria reduir les complicacions derivades de l'ictus i el tractament trombolític així com millorar el pronòstic del pacient, però en moments posteriors caldria abandonar aquesta inhibició i potser fins i tot estimular l'expressió de les MMPs per potenciar els processos de neurogènesi i vasculogènesi per reparar el teixit danyat.

1.7.3 La MMP-9 i les cèl·lules progenitores

Les cèl·lules mare a la medul·la òssia es troben en un estat de repòs i són activades per a diferenciar-se i mobilitzar-se cap a la circulació en resposta a senyals específics. Com hem vist anteriorment, en l'edat adulta la medul·la òssia és un important reservori de cèl·lules mare específiques d'òrgans, incloent cèl·lules mare hematopoètiques, progenitores endotelials, o cèl·lules mare mesenquimals (157). Durant l'estat estacionari, la majoria de les cèl·lules mare estan en contacte amb les cèl·lules estromals de medul·la òssia, com els osteoblasts, i es mantenen en la fase G0 del cicle cel·lular, mentre que una petita fracció es troba en fase S o G2/M (158). L'equilibri entre aquestes fraccions està dictat per la biodisponibilitat de citocines activadores, que es troben a la matriu extracel·lular o estan lligades a la membrana de les cèl·lules estromals. Quan les cèl·lules han de ser mobilitzades, es produeix un alliberament de citocines i quimiocines que promouen aquest alliberament i migració cel·lular. Algunes cèl·lules mare i progenitores, com les endotelials, expressen c-kit, el receptor del lligand Kit (KitL), suggerint que una cascada de senyalització comú pot governar la seva proliferació i reclutament (158,159). La MMP-9 és clau en aquest procés d'alliberament i reclutament de les cèl·lules. L'augment en l'expressió de MMP-9 després d'alguns processos d'estrès cel·lular, activa l'alliberació de KitL que, per la seva banda, facilita la mobilització de les cèl·lules de la medul·la òssia a la circulació i alhora activa el cicle cel·lular de les cèl·lules mare hematopoètiques (119).

Un estudi previ va demostrar que ratolins deficients en MMP-9 van mostrar una disminució de la neovascularització en un model d'isquèmia perifèrica (160). Aquest fet s'acompanyava d'una menor mobilització de les EPCs després de la isquèmia en els animals *knock out* (KO) (161). Aquesta mateixa reducció de la vascularització en l'extremitat isquèmica es produïa en animals deficients en MMP-2. La deficiència de MMP-2 es relacionava a més amb una disminució del nombre d'EPCs i de la seva funcionalitat *in vitro* (162). En el cas de la isquèmia cerebral no s'ha descrit encara la relació entre al deficiència de MMPs i la mobilització de cèl·lules mare o progenitores com les EPCs.

La relació entre la MMP-9 i les cèl·lules mare o progenitores també apareix a nivell local al cervell. Per exemple, durant el procés de neurogènesi derivat de la isquèmia cerebral, diverses quimiocines, com el SDF-1 o el VEGF, promouen la migració dels progenitors neurals des de les zones subventriculars germinals cap a la perifèria de l'infart. Aquesta migració requereix la remodelació de la matriu extracel·lular, i l'expressió endògena de MMP-9 i MMP-3 en les cèl·lules neurals progenitores és necessària tant per la migració com la diferenciació d'aquestes cèl·lules (153).

2. OBJECTIUS

Els objectius principals d'aquesta tesi són:

1) Realitzar una revisió sistemàtica de la literatura sobre la implicació de les MMPs en el dany cerebral i en els processos de neuroreparació després de l'ictus, així com dels tractaments amb inhibidors de les MMPs testats en la fase aguda de l'ictus.

2) Comparar el potencial terapèutic de les EPCs i dels factors de creixement secretats per aquestes cèl·lules com a teràpia neuroreparadora en un model animal d'isquèmia cerebral.

3) Determinar l'efecte de la isquèmia cerebral en un model murí en l'alliberament i la funcionalitat *in vitro* de les EPCs.

4) Determinar la implicació de les MMPs, i específicament la MMP9, en la funcionalitat *in vitro* de les EPCs murines i humanes.

5) Desenvolupar un nou mètode d'isquèmia cerebral transitòria en ratolí mitjançant l'oclusió temporal de l'artèria cerebral mitja a nivell distal.

3. RESULTATS

Article 1

Factors Secreted by Endothelial Progenitor Cells Enhance Neurorepair responses after Cerebral Ischemia in Mice.
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PLOS | ONE

Factors Secreted by Endothelial Progenitor Cells Enhance Neurorepair Responses after Cerebral Ischemia in Mice

Anna Rosell[1]*, Anna Morancho[1], Miriam Navarro-Sobrino[1], Elena Martínez-Saez[2], Mar Hernández-Guillamon[1], Silvia Lope-Piedrafita[3,4], Verónica Barceló[1], Francesc Borrás[2], Anna Penalba[1], Lidia García-Bonilla[1], Joan Montaner[1]

1 Neurovascular Research Laboratory, Department of Neurology and Department of Internal Medicine, Universitat Autònoma de Barcelona, Vall d'Hebron Research Institute, Barcelona, Catalonia, Spain, **2** Neuropathology Unit, Department of Pathology, Hospital Vall d'Hebron, Barcelona, Catalonia, Spain, **3** Servei RMN, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Catalonia, Spain, **4** Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Cerdanyola del Vallès, Catalonia, Spain

Abstract

Cell therapy with endothelial progenitor cells (EPCs) has emerged as a promising strategy to regenerate the brain after stroke. Here, we aimed to investigate if treatment with EPCs or their secreted factors could potentiate angiogenesis and neurogenesis after permanent focal cerebral ischemia in a mouse model of ischemic stroke. BALB/C male mice were subjected to distal occlusion of the middle cerebral artery, and EPCs, cell-free conditioned media (CM) obtained from EPCs, or vehicle media were administered one day after ischemia. Magnetic resonance imaging (MRI) was performed at baseline to confirm that the lesions were similar between groups. Immunohistochemical and histological evaluation of the brain was performed to evaluate angio-neurogenesis and neurological outcome at two weeks. CM contained growth factors, such as VEGF, FGF-b and PDGF-bb. A significant increase in capillary density was noted in the peri-infarct areas of EPC- and CM-treated animals. Bielschowsky's staining revealed a significant increase in axonal rewiring in EPC-treated animals compared with shams, but not in CM-treated mice, in close proximity with DCX-positive migrating neuroblasts. At the functional level, post-ischemia forelimb strength was significantly improved in animals receiving EPCs or CM, but not in those receiving vehicle media. In conclusion, we demonstrate for the first time that the administration of EPC-secreted factors could become a safe and effective cell-free option to be considered in future therapeutic strategies for stroke.

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* E-mail: anna.rosell@vhir.org

Introduction

Stroke remains a major cause of death and disability worldwide. However, the only approved treatments are pharmacological and mechanical reperfusion therapies, which aim to restore blood flow in hyperacute ischemic patients [1]. Although thrombolysis with tissue plasminogen activator is effective and lifesaving, only 2–5% of all stroke patients receive this treatment. Therefore, it is necessary to develop new stroke therapies that could be used to treat a large number of patients in the delayed phases of this devastating disease. In this regard, the idea that neurovascular plasticity could contribute to brain recovery has emerged as a powerful new concept for stroke therapy [2]. Thus, for brain repair after stroke both angiogenesis and neurogenesis will have to be potentiated in the ischemic brain, and endothelial cells have been established as critical components of the neural stem cell niche. Indeed, they secrete soluble factors that maintain the neurogenic potential of the central nervous system [3] and endogenous angiogenesis has been causally linked to endogenous neurogenesis after stroke in mice [4].

Classically, the formation of new blood vessels was thought to be mediated exclusively by embryogenic vasculogenesis, followed by the sprouting of endothelial cells from preexisting vessels during angiogenesis [5]. However, this dogma was called into question upon the discovery of bone marrow-derived $CD34^+$ cells with endothelial characteristics and circulating in adult human blood [6]. These cells, referred to as endothelial progenitor cells (EPCs), were capable of differentiating in vitro into endothelial-phenotyped cells, and represent a new model for endothelial generation and vessel repair. Thereafter, multiple studies using both in vitro and in vivo models of angiogenesis have confirmed the role of these EPCs as an alternative cell-based approach to enhance angio- and vasculogenic responses. However, few studies have investigated their role in animal models of cerebral ischemia. In this regard, neovascularization and neuronal regeneration could be successfully induced after delayed systemic administration of human cord blood-derived $CD34^+$ containing EPCs in a mouse model of cerebral ischemia [7]. Another study showed that the hyperacute transarterial administration of bone marrow-derived EPCs expanded ex vivo reduced both infarct volume and neurological deficit in a focal ischemia-reperfusion rat model, by attenuating endothelial dysfunction [8]. More recently, it was shown that the administration of endothelial colony-forming cells co-cultured with EPCs one day after transient cerebral ischemia improved functional recovery by reducing the number of apoptotic cells and increasing brain angiogenesis in rats [9]. Finally, other authors have suggested that the hyperacute administration of EPCs protects the brain against ischemic injury and promotes neurovascular repair, thus improving long-term neurobehavioral outcome through SDF-1-mediated signaling pathways [10]. However, when translated to clinical practice, cell-based therapies may lead to adverse side effects such as microembolism or tumor formation, lung dysfunction, or abnormal immune system reactions [11,12]. In this context, it has been proposed that cell-free, but cell-based, strategies could open new avenues in the field of regenerative medicine and should be explored for stroke treatment [13].

To the best of our knowledge, the potential therapeutic benefits of EPC-secreted factors as cell-free therapies have not been investigated in preclinical models of stroke. Our hypothesis is that a cell-free treatment based on the administration of paracrine factors secreted by EPCs could enhance neurorepair after cerebral ischemia. Therefore, the aim of this study was to demonstrate for the first time that angiogenic treatment with EPCs, or conditioned media (CM) containing EPC-secreted factors, could potentiate cerebral angiogenesis and improve neurorepair after stroke.

Materials and Methods

Ethics Statement

The Ethics Committee of Animal Experimentation (CEEA) of Vall d'Hebron Research Institute approved the study protocol (No. 20/09) and all experiments were conducted in accordance with the Spanish legislation and the Directives of the European Union. Metamizol magnesium (300 mg/kg) was administered subcutaneously after the procedure to prevent surgery-related pain. The ARRIVE guidelines were considered when designing and reporting the results of the study.

Endothelial Progenitor Cell Culture

Previously characterized EPCs were obtained as described elsewhere [14]. Briefly, a pool of spleens obtained from BALB/C mice males (Charles River Laboratories, Spain) was mechanically minced, placed at 37°C for 15 minutes in 2 mM EDTA and run through a 40-μm nylon membrane to obtain a cell suspension. Mononuclear cells (MNCs) were obtained by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Sweden), shortly washed with a red blood cells lysis solution (150 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$ and 0.1 mmol/L EDTA in distilled water) and gently washed with complete endothelial growth medium-2 (EGM-2, Lonza, USA). The medium is composed of endothelial cell basal medium (EBM) containing 5% fetal bovine serum, human endothelial growth factor (hEGF), vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (hFGF-B), insulin-like growth factor-1 (R3-IGF-1), GA-1000 (gentamicin and amphotericin-B), heparin, hydrocortisone and ascorbic acid. Isolated MNCs were finally resuspended in EGM-2 and 5×10^6 MNCs were seeded on fibronectin-coated flasks (25 cm^2) and incubated in a 5% CO_2 atmosphere at 37°C. Under daily observation, first media change was performed two days after plating and thereafter, media was changed every 2–3 days.

Permanent Focal Cerebral Ischemia

Adult BALB/C mice males weighing 25–30 g (Charles River Laboratories) were given free access to food and water prior to surgery. A reproducible model of stroke by middle cerebral artery occlusion (MCAO) affecting the cortex was induced by electrocauterization of the distal portion of the left MCA as previously described [15]. Metamizol magnesium (100 mg/kg) was administered subcutaneously right after the procedure as analgesia. The duration of anesthesia in all animals was less than 30 minutes. Mice that showed a decrease in cerebral blood flow (>75%) after the procedure as compared to baseline values were used for experiments. Sham surgery consisted of the same procedure, except that the artery was not electro-coagulated. A total of 44 mice were initially included in the study (40 MCAO and 4 shams). The animals were euthanized at two weeks.

In Vivo Magnetic Resonance Imaging (MRI)

MRI was performed at a 7T horizontal magnet system (BioSpec 70/30 USR, Bruker BioSpin, Ettlingen, Germany) equipped with actively shielded gradients capable of 400 mT/m (B-GA12 gradient coil inserted into a BGA20S gradient system) and a dedicated mouse brain quadrature surface coil, actively decoupled from a transmit volume coil with 72-mm inner diameter. The animals were positioned on a mouse bed, which allowed for a localized delivery of anesthesia (isoflurane, 1.5–2.5% in O_2 at 0.8 L/min); respiratory frequency was monitored with a pressure probe and maintained at 60–80 breaths/min. A water recirculating system integrated into the animal bed was used to control the body temperature, which was measured with a rectal probe (37±1°C).

T2-weighted fast spin-echo images (T2WIs) were initially obtained in axial, sagittal and coronal planes to be used as reference scout images for reproducible slice selection at each MRI session. Imaging parameters for these images were: echo time (TE) = 12 ms, echo train length (ETL) = 8, effective echo time (TEeff) = 36 ms, repetition time (TR) = 3 s, field of view (FOV) = 2.4×2.4 cm^2, matrix size (MTX) = 256×256, and slice thickness (ST) = 1 mm. Afterward, coronal MRI sections were acquired over an 8.7 mm block starting 1.5 mm anterior to the bregma and towards the cerebellum. T2WIs were acquired using a fast spin-echo sequence with ETL = 8, TEeff/TR = 60 ms/4.2 s, FOV = 2.0×2.0 cm^2, MTX = 256×256, 18 contiguous slices with ST = 0.5 mm and a 0.1-mm gap between them. T2 maps were also obtained using a multi-slice multi-echo sequence with 20 TE values ranging from 10 to 200 ms, TR = 4 s, MTX = 128×128, FOV = 2.0×2.0 cm^2, and slices covering exactly the same brain region as in high resolution T2W coronal images, but with eight continuous 1-mm slices and 0.1 mm gaps. Diffusion tensor imaging (DTI) was carried out using a spin echo 4-shot echo-planar readout sequence with identical geometries as in T2 mapping. Diffusion-weighted images were acquired along 30 diffusion directions and 3 b-values (b = 0, 500, 1000, 2000 s/mm^2). The imaging parameters were: TE/TR = 35 ms/3 s, diffusion gradient duration (δ) = 4 ms, diffusion gradient separation (Δ) = 20 ms, MTX = 64×64, FOV = 2.0×2.0 cm^2. Maps of the trace of the diffusion tensor, also referred to as apparent diffusion coefficient (ADC), and fractional anisotropy (FA) were derived using ParaVision 5.0 (Bruker BioSpin, Ettlingen, Germany) as described previously [16]. The total preparation and scan time for each animal was approximately of one hour.

A total of 38 animals were scanned 48 hours after ischemia (34 MCAO and 4 shams) before any treatment was given. Two mice died during MRI related to uncontrolled body temperature and four mice were excluded from the study for undersized infarcts (probably caused by electrocoagulation of only one of the branches of the distal portion of the MCA). Six animals included in the study were not scanned, because the MR scanner was not available at the last moment. A group of 15 mice were scanned two weeks after ischemia or sham surgery to monitor lesion extension and severity. However, since a complete necrosis and atrophy of the injured cortex was observed in ischemic mice (shown in Figure 1A) the following animals did not received a second scan.

Regions of interest (ROIs) of 1 mm^2 were traced on T2, ADC and FA maps in all slices presenting infarcts (and inside the injured area). Measures of the corresponding contralateral hemisphere were also obtained. Finally, mean values were expressed as a ipsilateral/contralateral ratio, as described by others [17,18].

Treatment Groups

The neurorepair potential of different treatments was tested here in animals, to assess the possibility of giving them to stroke patients beyond the hyperacute phase. In order to avoid any potential confounding effect due to neuroprotection, treatments were administered one day after ischemia. Briefly, growing EPCs were washed twice at day 5–6 with EBM. Then, 4 mL of fresh EBM were added to obtain CM that was collected 24 hours later. CM was concentrated for 40 min using a 10 kDa filter unit (Millipore, Ireland), for a final volume of approximately 200 µL. At the same time, EPCs were trypsinized, counted and resuspended in 200 µL of EBM. Treatments were administered intravenously using a 30 G needle as follows: vehicle (n = 11, 200 µL of EBM), EPCs (n = 11, 200 µL of cells in EBM), CM (n = 12, 200 µL of CM). Sham animals received 200 µL of EBM (n = 4). The total number of EPCs administered ranged from 10^4

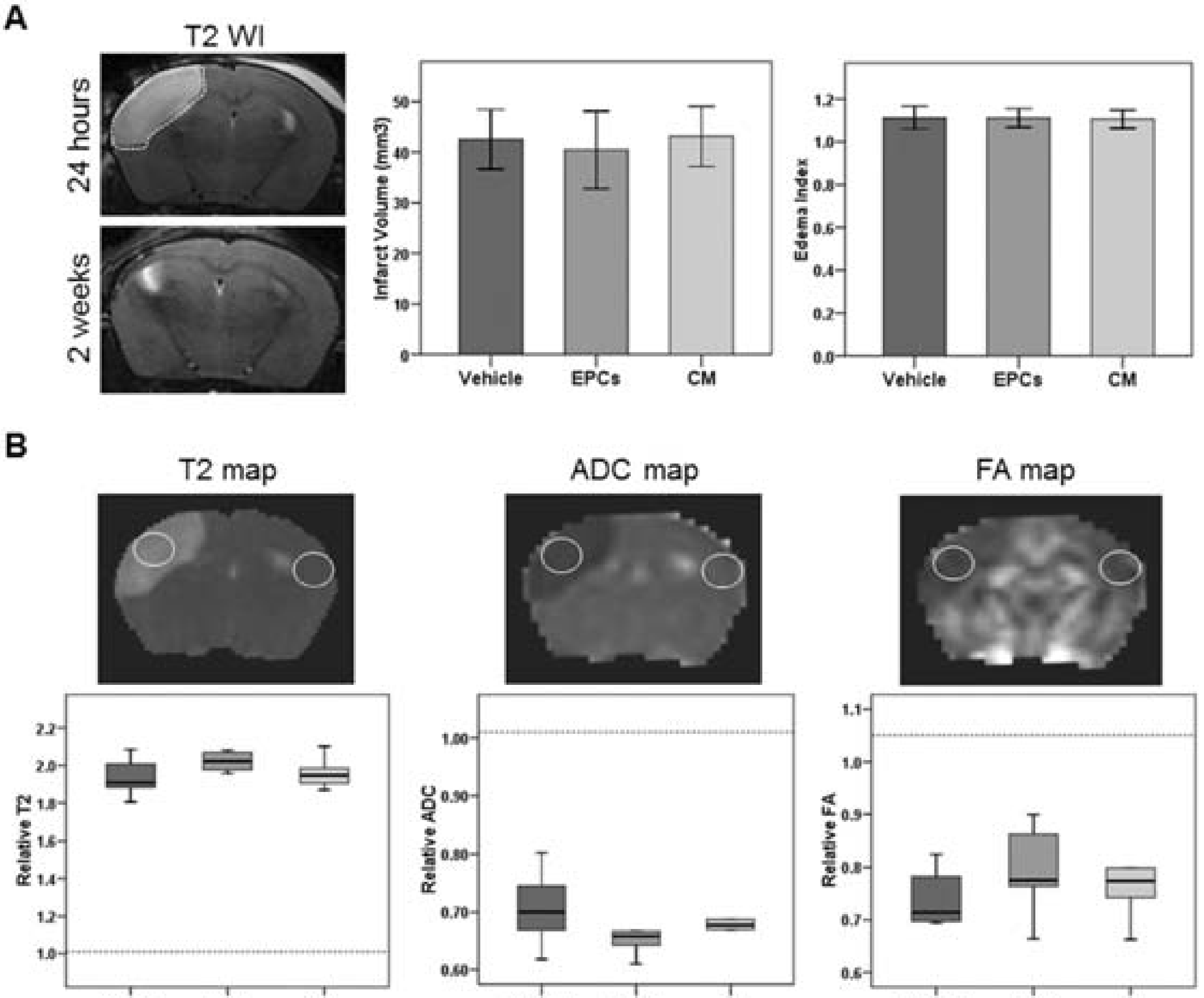

Figure 1. MRI after cerebral ischemia. Representative T2WI used to measure infarct volume at 24 hours (upper image, dashed lines in white) and representative T2WI of the brain at two weeks showing severe cortical damage; bar graphs represent infarct volume and edema index at 24 hours (**A**); data is presented as mean ± SD. Representative T2, ADC and FA maps of an ischemic animal and box-plots representing relative values (related to contralateral values) of ROIs; dashed line shows reference (median) for sham animals (**B**); data is expressed as median (IQR). Number of animals: n = 9 for vehicle, n = 9 for EPCs and n = 9 for CM and n = 3 for shams. No difference was found between ischemic groups.
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to 1×10^5; since different cell cultures yield different number of EPCs, this allowed us to investigate any association between the amount of administered cells and angiogenesis or recovery. Treatments were administered randomly at 30–34 hours post-ischemia after the first MRI session, and all animals received intraperitoneal injections of 5-bromo-2'-deoxyuridine (BrdU, Sigma, MO, USA, 50 mg/Kg), starting five days after ischemia and until sacrifice. One animal receiving CM treatment died a week after surgery.

The same surgical procedure was performed in a separate group of animals (n = 12) to analyze physiological variables. Treatments were administered intravenously 30 hours after ischemia as follows: vehicle (n = 3, 100 µL of EBM), EPCs (n = 3, 100 µL of cells in EBM), CM (n = 3, 100 µL of CM) and sham group (n = 3, 100 µL of EBM). Thirty minutes after treatment, cardiac arterial blood was drawn from the left ventricle and pH, blood gases (pO_2 and pCO_2), glucose, hemoglobin and electrolytes (Na^+, K^+ and HCO_3^-) were monitored using the i-STAT Portable Clinical Analyzer (Abbot). These animals all met the LDF criteria on arterial occlusion and reperfusion. Then, the brain was removed and the infarct volume measured using 2.5% of 2,3,5-triphenyl-tetrazolium chloride staining as described previously [15], to confirm the extent of the infarct.

Infarct Volume

Infarct volume and brain edema were determined using Image J. The hyperintense areas corresponding to infarcted tissue and the complete areas of both ipsilateral and contralateral hemispheres were traced manually on T2WIs that were obtained one day after cerebral ischemia (16 slices/brain) by researchers who were blinded to treatment. The value for each slice was corrected for section thickness (0.55 mm). An edema index was determined as the sum of all areas of the ipsilateral hemisphere divided by the sum of all areas of the contralateral hemisphere. Lesion volume was calculated as the sum of the infarcted area across all slices and corrected for edema as proposed by our group and others [15,19]. Results were given in mm³.

Behavioral Testing

Several tests were used to assess stroke severity and functional outcome before MCAO (pre-MCAO), at 48 hours, at one week and at two weeks. Initially, a modified neurological severity score was used to assess stroke severity and functional outcome [20]. However, the test failed in demonstrating an impairment of function at 48 hours in most animals, which led us to use another test. Therefore, the corner test and the grip strength meter were used to evaluate more specific neurological functions as described elsewhere [8]. Briefly, the corner test used to assess sensorimotor and postural asymmetries after MCAO consisted of two boards (30 cm×20 cm×1 cm) attached at a 30° angle with a small opening between the boards to encourage entry in the corner. Ten trials were performed per animal and left and right turns were recorded. A laterality index was calculated as described previously [15,21]. The grip strength meter measures the maximum strength exhibited by the mouse forelimbs [8]. Briefly, animals were suspended by the tail and approached to the grid. Once the mouse grasped the center of the grid with both forelimbs the animal was pulled backwards in the horizontal plane. The force applied to the grid was recorded as the peak tension and measured in grams. Six measures were acquired per time point and the mean force was obtained for statistical analysis. Researchers who were blinded to treatment group performed the functional tests.

Immunohistochemistry

Two weeks after ischemia mice were deeply anesthetized with isoflurane, the brains were transcardially perfused with 4% paraformaldehyde and fixed overnight at 4°C. Afterwards, brains were cryoprotected with 30% sucrose in PBS for 48 hours and frozen in isopentane and embedded in optimal cutting temperature (OCT) before storage at −80°C. Twelve µm-thick coronal sections (n = 9) were obtained serially, starting after the olfactory bulb, and every 400 µm. Immunostaining with antibodies for endothelial cells (Lectin, Vector Labs, USA or CD-31, Abcam, UK), proliferating cells (BrdU, Abcam, UK), mature neurons (NeuN; Millipore, UK) and neuroblasts (doublecortin, DCX, Abcam, UK) was performed as follows: sections were thawed at room temperature for 30 minutes and rehydrated in PBS for 10 minutes. Then, cells were permeabilized with 0.5% triton X-100 for 10 minutes and blocking buffer (1% BSA and 5% goat serum) was applied for one hour. Primary and secondary antibodies were applied in blocking buffer and slides were finally mounted in Vectashield® with DAPI to counterstain cell nuclei. BrdU-stained sections were pretreated with 2N HCl for 30 min to open the DNA structure and neutralized with 0.1M borate buffer (pH 8.5) before incubation in blocking buffer. Vessel density and neurons were measured in peri-infarct cortical areas adjacent to the damaged tissue using Image J. Briefly, three slices separated by a same distance were stained for lectin or NeuN. Four images of the peri-infarct and contralateral cortex were captured with an inverted fluorescent microscope at 400× (Olympus, IX71). Finally, a total of 12 fields including different areas were used to compute the mean vessel density by a researcher who was blinded to treatment. Data were corrected by the signal of the corresponding contralateral hemisphere and expressed as a ratio. DCX and BrdU co-staining were only performed in sections including the subventricular zone (SVZ).

Axonal Fiber Histology

Axonal fiber pathways were examined using Bielschowsky's staining method. Briefly, slides were placed in 20% silver nitrate in the dark, and then ammonium hydroxide was added until the tissue turned brown with a yellow background. Slides were then washed in 95% ethanol, absolute ethanol and xylene, and then mounted in mounting medium. This stain shows axons in black. Positive stain was used to measure the thickness of the axon track emerging from the corpus callosum (CC) at the level of the infarct boundary by a neuropathologist who was blinded to treatment. The same measure was taken from the contralateral structure at the same level for data normalization. Thickness was expressed as a ratio between the ipsilateral and contralateral hemispheric measures.

Multiplex ELISA

Additional EPC-secreted CM was obtained as described above through six independent EPC cultures. After concentration, CM was stored at −80°C until use. The content of EPC-secreted growth factors was determined using SearchLight® Angiogenesis Array 1 (Aushon Biosystems, MA, USA) for the simultaneous measurement of four angiogenic factors: PDGF-bb, HGF, FGF-b and VEGF. Cross-reaction with mouse proteins was confirmed for VEGF and PDGF-bb, whereas no data was available for HGF or FGF-b. Samples were assayed twice and the mean value of the two measurements was calculated. The mean intra-assay coefficients of variation were <20%. The images were analyzed with Array Analyst (Aushon Research, USA). The sensitivity limits were 2.0 pg/ml for PDGF-bb, 3.2 pg/ml for HGF, 2.0 pg/ml for FGF-b, and 4.0 pg/ml for VEGF. Importantly, concentrated EBM was also tested as a blank sample. Additionally, a Bradford assay was performed to determine total protein concentration and the values were normalized to pg of factor/µg of protein. Assays were performed by personnel blinded to treatment.

Statistical Analysis

SPSS 15.0 was used for all statistical analyses. Shapiro-Wilk or Kolmogorov-Smirnov tests were used to verify the normal distribution of the variables. Statistical significance for intergroup differences for normally distributed variables was assessed using Student's t-test or ANOVA followed by Fisher's least significant difference (LSD) test. Non-normally distributed variables were analyzed using Kruskall-Wallis and Mann-Whitney tests. Repeated measure ANOVA was used to assess differences in functional recovery between time-points. Correlations were evaluated using Pearson's coefficient for normally distributed variables. A p-value <0.05 was considered statistically significant at a 95% confidence level. Values from normally distributed variables were expressed as mean ± SD and represented as bar graphs, whereas values from non-normally-distributed variables were expressed as median (interquartile range) and represented as box-plots.

Results

Quantitative Characterization of MRI Parameters of Brain Injury

Infarct volume was similar in all groups at 24 hours of ischemia and before any treatment was administered (vehicle: [illegible] mm^3, EPCs: 40.1±7.6 mm^3 and CM: [illegible] mm^3) as shown in Fig. 1A. Sham animals had negligible lesions ([illegible] mm^3), probably due to the trauma of surgery. The edema index was also similar between ischemic groups at 24 hours (vehicle: [illegible]; EPCs: [illegible] and CM: [illegible]; Fig. 1A) whereas the reference was [illegible] in sham animals. No difference was found in infarct volume or brain edema between ischemic groups, whereas all of them were significantly different when compared to the sham group ($p<0.05$). Severe lesions were observed at two weeks with a complete atrophy of the lesioned cortex (Fig. 1A, bottom image).

Measurements of T2, ADC and FA maps within the infarct core confirmed that the severity of the lesion was similar between all ischemic groups before any treatment was given (Fig. 1B). High T2 values were found to be similar between all ischemic groups: [illegible] for vehicle, [illegible] for EPCs and [illegible] for CM, while the reference for sham animals was [illegible]. At the same time, low ADC values were detected in infarct areas of all ischemic groups: 0.70 (0.65–0.75) for vehicle, 0.65 (0.62–0.71) for EPCs and 0.67 (0.65–0.70) for CM, while the reference for sham animals was [illegible]. Finally, relative FA values were below the sham reference [illegible] and similar between ischemic groups: [illegible] for vehicle, [illegible] for EPCs and [illegible]. No difference was seen among ischemic groups for any of the measured parameters at 24 hours and all of them were significantly different compared to the sham values ($p<0.05$), indicating similar ischemic lesions (Fig. 1B).

Physiological Variables

The physiological variables measured in arterial blood 30 minutes after treatment administration remained unaltered among groups (see Table S1) and infarct volume was similar between these MCAO groups (vehicle: [illegible] mm^3, EPCs: [illegible] mm^3 and CM: [illegible] mm^3; $p>0.05$).

Effects of EPC and Cell Free Secreted Factors on Cortical Angiogenesis

Angiogenesis was considerably enhanced in the cortical peri-infarct areas of animals receiving pro-angiogenic treatment. Our results show that vessel density was significantly increased at peri-infarct tissue at two weeks in EPC- and CM-treated animals when compared to vehicle treatment ($p<0.001$ and $p<0.0001$, respectively) and to sham-operated animals ($p=$ [illegible] and $p=$ [illegible], respectively), as shown in Fig. [illegible]A. Representative micrographs of peri-infarct lectin positive vessels are shown in Fig. [illegible]C. Double positive staining of CD-31 endothelial cells with BrdU was also seen in these peri-infarct areas as shown in Fig. [illegible]D. Regarding the administered CM containing EPCs secretome, several growth factors were identified including VEGF (vascular endothelial growth factor; [illegible] pg/µg of total protein), FGF-b (fibroblast growth factor basic; [illegible] pg/µg of total protein) and PDGF-bb (platelet-derived growth factor; [illegible] pg/µg of total protein) as shown in Fig. [illegible]B. We were not able to detect HGF (hepatocyte growth factor) in the analyzed CM. Finally, no correlation could be found between vessel density and the number of administered EPCs or dosed CM ($r=$ [illegible] and $r=$ [illegible], respectively; $p>0.05$).

Axonal Reorganization and Neuroblast Migration

Histological examination revealed signs of axonal reorganization, as white matter tracks emerging from the corpus callosum were thicker at the level of the peri-infarct cortex in ischemic animals (Fig. 5A, B). The corpus callosum was thicker, with axons leaving the callosal track towards the peri-infarct, in animals that received EPCs than shams ([illegible] vs. [illegible]; $p=$ [illegible]), but not in vehicle-treated or CM-treated animals (Fig. 5B). No difference could be found when comparing ischemic animals ([illegible] for vehicle, [illegible] for EPCs and [illegible] for CM; $p>0.05$). Moreover, no correlation was found between corpus callosum thickness and number of administered EPCs or dosed-CM ($r=$ [illegible] and $r=$ [illegible], respectively; $p>0.05$) and no difference was detected between groups for contralateral measures ($p>0.05$).

DCX-positive cells, a marker for neurogenesis, were identified in the dorsolateral SVZ zone of sham animals. However, in response to ischemia, the DCX-positive signal increased by migration of neuroblasts towards peri-infarct regions, as shown in the image composition in Fig. 6A, which represents an EPC-treated animal. Migrating DCX-positive neuroblasts in the SVZ were particularly visible in brains from animals receiving CM treatment (Fig. 6B) and co-staining with BrdU indicated that most of these cells were also under proliferation, as seen in Fig. 6B. However, the amount of neuroblasts expressed as DCX-positive area in the SVZ was not different between groups: [illegible] for vehicle, [illegible] for EPCs, [illegible] for CM and [illegible] in shams ($p>0.05$) as shown in Fig. 6C. The BrdU+ area in those SVZ were slightly increased in animals receiving EPCs: [illegible] for vehicle, [illegible] for EPCs, [illegible] for CM ($p>0.05$) as shown in Fig. 6C.

The presence of migrating neuroblasts was clearly seen in the border of the infarct area (Fig. 6A) but not in the contralateral cortex. In peri-infarct areas, DCX-positive cells were not under proliferation, as BrdU-positive cells did not match with DCX signal. Cell counts for NeuN-positive mature neurons in peri-infarct areas did not reveal any significant difference among treatments ($p>0.05$).

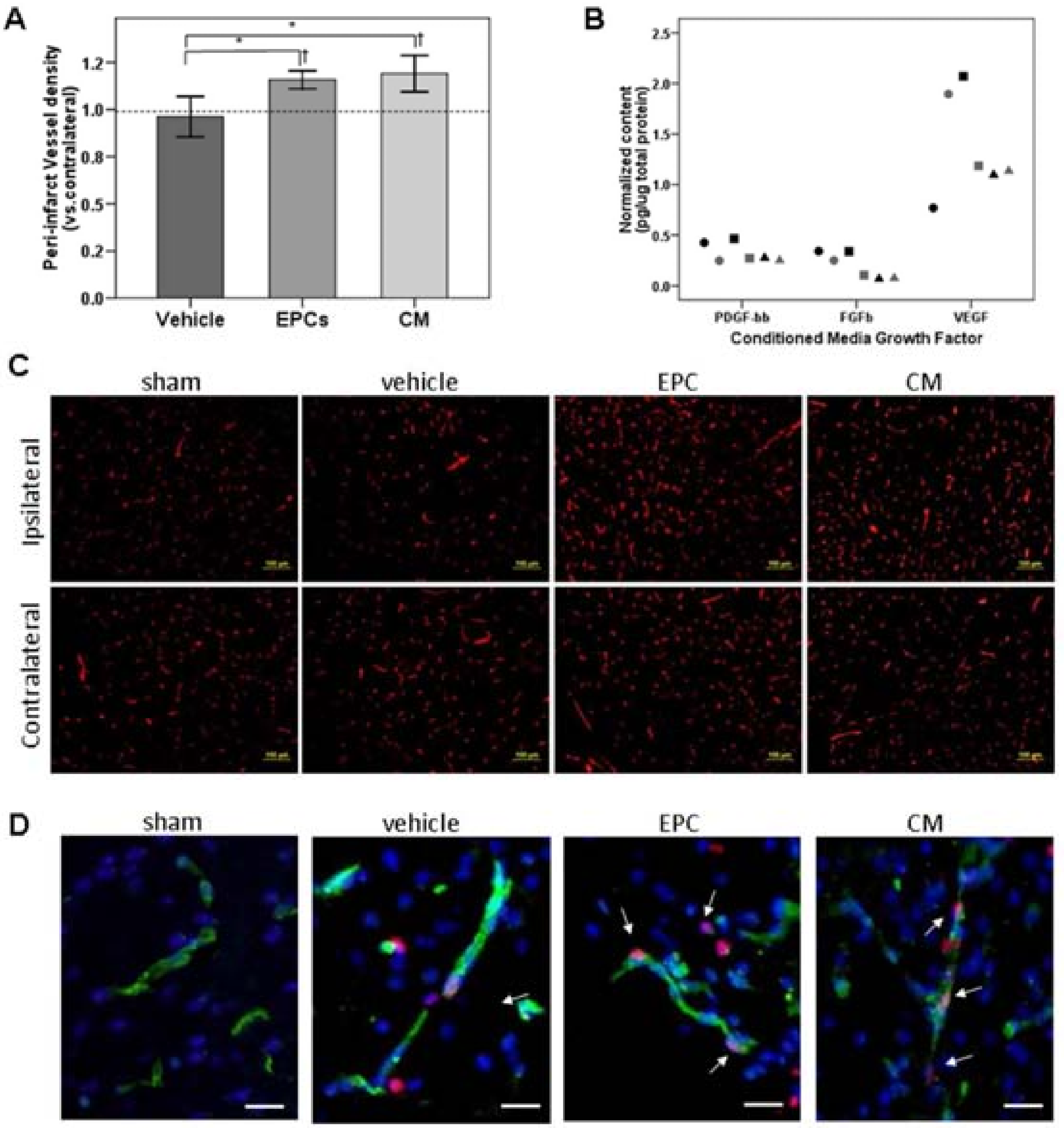

Figure 2. Peri-infarct angiogenesis after treatment. Bar graph representing vessel density (mean±SD vs. contralateral) in peri-infarct regions of ischemic animals treated with vehicle (n = 10), EPCs (n = 9) or CM (n = 6); dashed line shows reference (mean) for sham (n = 4) animals (**A**); * indicates $p<0.05$ vs. vehicle and † indicates $p<0.05$ vs. sham. Graph showing CM content of PDGF-bb, FGF-b and VEGF in six different cultures of EPCs represented as black or grey circles, squares or triangles (**B**). Representative micrographs of lectin immunofluorescence; bars represent 100 µm (**C**). Representative micrographs of CD-31 (green) and BrdU (red) staining merged with DAPI showing proliferating endothelial cells (arrows) in peri-infarct areas; bars represent 20 µm (**D**).

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EPC-based Pro-angiogenic Therapy Improves Neurological Outcome

The grip strength meter test identified neurological deficits 24 hours after ischemia in all groups (n = 6/group) when compared to pre-MCAO values (post 82.4±13.2 vs. pre 111.1±17.2 for vehicle, p = 0.044; post 84.2±6.8 vs. pre 116.1±11.3 for EPCs, p = 0.002; post 83.5±8.7 vs. pre 105.3±8.2 for CM; P = 0.027).

Importantly, the forelimb strength of animals receiving EPCs or CM treatment was improved at one week and two weeks, as compared to the 24 h post-ischemia score (1 w 94.2±6.5 and 2 w

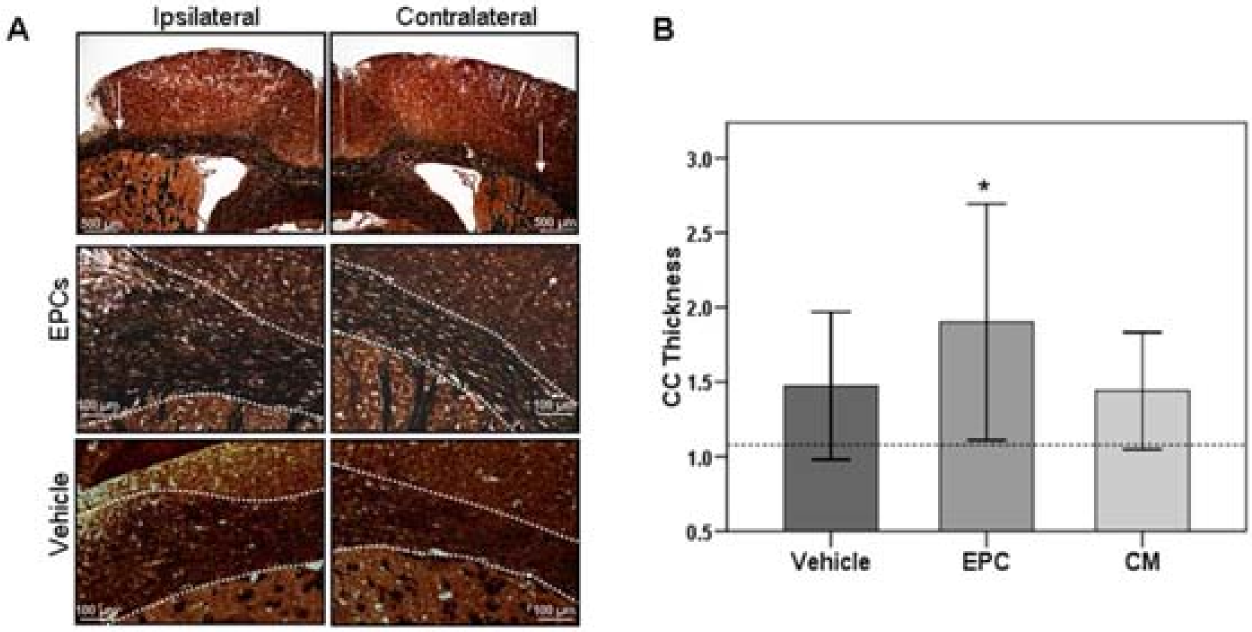

Figure 3. Representative images of Bielschowsky's staining. Ipsilateral and contralateral hemispheres of vehicle and EPC-treated brains (**A**); bars represent 500 µm. White arrows in (**A**) indicate the region of the corpus callosum (CC) with increased thickness, with emerging axonal tracks shown in detail in the bottom panels. Bar graph representing the ipsilateral CC axonal thickness expressed as a ratio vs. the contralateral hemisphere ($n = 10$ for vehicle, $n = 9$ for EPCs and $n = 7$ for CM), dashed line shows reference (mean) for sham ($n = 4$) animals (**B**). Data is presented as mean±SD. * indicates $p < 0.05$ vs. sham.
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103.7±8.0 vs. post 84.2±6.8, $p = 0.017$ and $p = 0.003$ for EPCs; 1 w 92.8±10.1 and 2 w 91±10.9 vs. post 83.5±8.7, $p = 0.049$ and $p = 0.026$ for CM). Although spontaneous recovery could be observed in some vehicle-treated animals over time, this improvement was not significant for this group (1 w 99±16.3 and 2 w 97.4±17.7 vs. post 83.8±12.2; $p = 0.226$ and $p = 0.302$, respectively). The neurological score test failed to demonstrate any impairment in neurological function after ischemia in most animals, although large lesions could be observed by MRI. The corner test only showed neurological impairment one day after ischemia in the cohort of vehicle animals, when compared to pre-MCAO scores ($p = 0.043$). It failed to show impairment after ischemia in EPC- and in CM-treated groups ($p = 0.157$ and $p = 0.273$, respectively), although large lesions could be observed by MRI.

Discussion

The present study confirms the angiogenic potential of EPC-secreted factors to safely treat cerebral ischemia beyond the hyperacute phase in a cell-free approach. Indeed, in a mouse model of stroke, we demonstrated that both EPCs and EPC-cell-free treatments significantly increased angiogenesis in peri-infarct areas where neurorepair should be potentiated. With regard to neurogenesis, our study could only demonstrate mild effects of the tested EPC-based treatments. Functional improvement at two weeks was also confirmed to be enhanced in mice receiving EPCs or CM. To the best of our knowledge, this is the first study to investigate the therapeutic potential of EPC-secreted factors in an *in vivo* model of cerebral ischemia.

In recent years, there has been growing interest in developing new neurorepair strategies for the treatment of stroke and other cardiovascular diseases. The narrow time window for neuroprotection, the identification of endogenous repair processes in the damaged brain, and the technical advances to obtain and grow many types of stem and progenitor cells have provided new opportunities for stroke treatment. In fact, phase I and II clinical trials using intracerebral transplantation of an immature neuron cell line [22], or intravenous transplantation of autologous mesenchymal stem cells [23] have already been conducted in stroke patients. Other studies have already confirmed that the intra-arterial administration of autologous bone marrow-MNCs days or months after stroke was also safe and feasible [24,25]. However, to date, no study has proven the efficacy of these treatments on motor function or functional dependency in stroke patients.

Several studies have demonstrated the therapeutic potential of EPCs for the treatment of many ischemic diseases in experimental models. Regarding cerebral ischemia, Ohta et al. first demonstrated that the autologous intra-arterial transplantation of early bone marrow-derived EPCs after 90 minutes of ischemia reduced infarct volume and improved motor function [8]. In a mouse model of transient MCAO, Fan et al. showed again that the acute intravenous administration (at one hour) of human early EPCs was associated with a reduction in infarct volume and brain atrophy at day 3, a reduction in long-term neurological deficits, and an increase in vessel density [10]. More recently, it was reported for the first time that the administration of outgrowth populations of human EPCs improved neurological function in a rat model of ischemia-reperfusion. This improvement occurred along with an increase in angiogenesis, a decrease in apoptosis in peri-infarct areas and an increase in neurogenesis in the SVZ [9].

In accordance with these data, our results showed for the first time that the delayed administration of early EPCs (approximately 30 hours after permanent ischemia) could potentiate angio-vasculogenic responses in mice, considering that more vessels

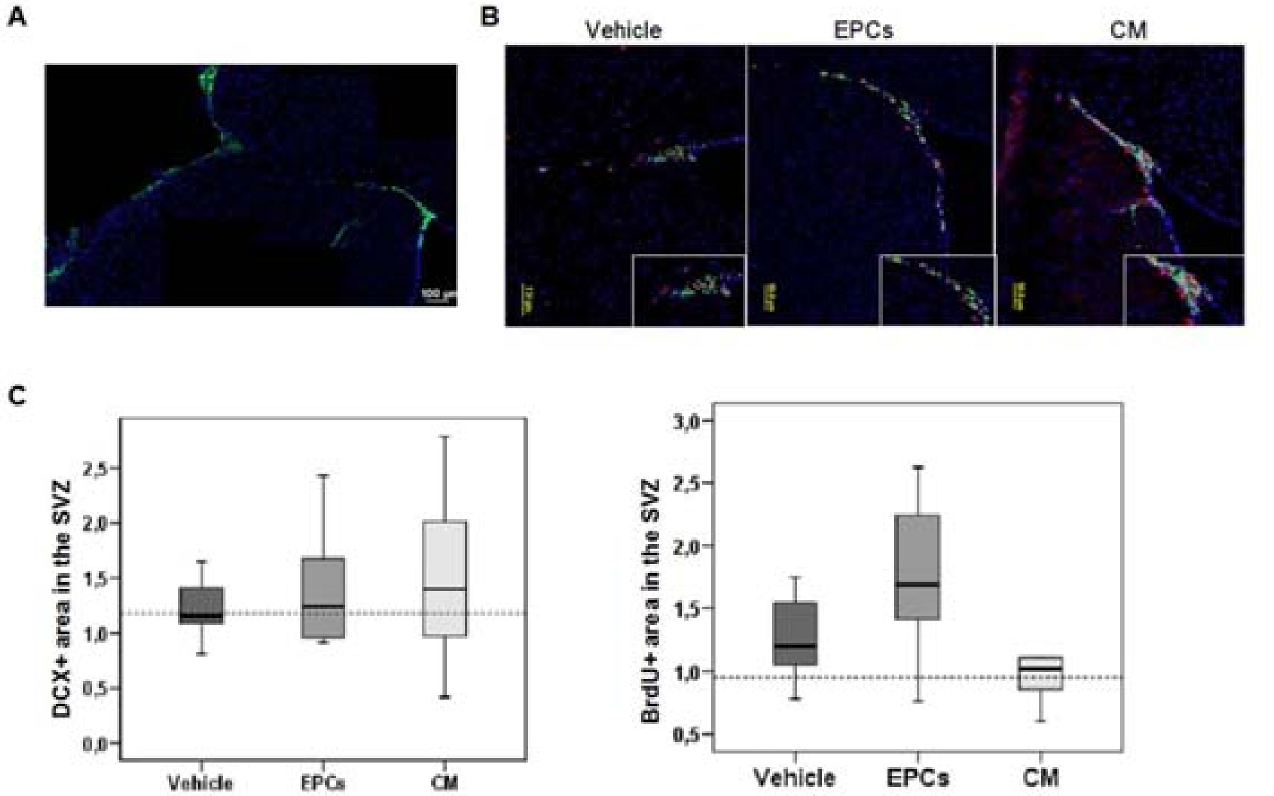

Figure 4. Immunofluorescence images showing neurogenesis at two weeks in the SVZ. Composition of four immunofluorescence images for DCX (green) and DAPI (blue) showing neuroblast migration from the SVZ towards cortical peri-infarct thorough the corpus callosum external capsule in an EPC-treated animal (**A**). Representative images of double immunofluorescence for DCX (green), BrdU (red) and DAPI (blue) are shown for the different treatment groups (**B**). Bottom right inserts show a magnification of the dorsolateral SVZ. Box-plots representing the percentage of DCX+ or BrdU+ area in the SVZ (n = 11 for vehicle, n = 11 for EPCs, n = 11 for CM and n = 3 for shams) (**C**); data is represented as median (IQR).
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were formed in the peri-infarct areas two weeks after ischemia. More evidence of ongoing angiogenesis in the peri-infarct tissue is the presence of proliferating endothelial cells as shown by BrdU staining. Other authors have reported EPC-related angiogenesis occurring at this time point after cerebral ischemia in both rats and mice [9,26]. No endogenous response could be observed in our Balb/c vehicle-treated animals in terms of angiogenesis after ischemia. This lack of response following a hypoxic stimulus has previously been described in Balb/c mice [27], and was explained by different strain responses following angiogenic triggers, or to a greater tolerance to hypoxia. Therefore, we believe that our capacity to enhance angiogenesis in Balb/c animals strengthens the importance of our proposed EPC-based therapies, since it is considered to be more difficult to stimulate angiogenic responses in this mouse strain.

This angiogenesis enhancement was even greater in animals receiving cell-free therapy with EPC-secreted factors. The exogenous administration of growth factors to potentiate angiogenesis has been proposed before to treat ischemic stroke. In fact, it has already been shown in animal models of stroke that the late (but not early) administration of growth factors such as VEGF enhanced angiogenesis in the ischemic brain by improving neurological recovery [28]. Also, HGF gene transfer reduced infarct volume and safely enhanced local angiogenesis, without disturbing the blood-brain barrier [29]. Our results showed that spleen-derived EPCs secrete important growth factors that improve angiogenesis, such as VEGF, FGF-b and PDGF-bb, and probably many others that have not been investigated in the present study, such as angiogenesis inhibitors. Other authors have also already postulated that a novel cell-free therapeutic approach for angiogenesis with *in vitro*-generated conditioned medium obtained from EPCs could be a potent alternative to progenitor cell therapies [30]. These authors demonstrated that an intramuscular injection of EPC-CM was as effective as cell transplantation for promoting tissue revascularization in a rat model of chronic hindlimb ischemia [30]. Similar results have been reported for the treatment of diabetic dermal wounds, were the injection of EPC-CM into wounded diabetic mice promoted wound healing and increased neovascularization to a similar extent to EPC transplantation [31]. In the present study, we demonstrated for the first time that angiogenesis was enhanced with the administration of EPC-CM in an animal model of stroke.

An important caveat in the cell transplantation field is that by administrating large amounts of cells (millions) systemically, preclinical studies have failed to clearly show massive stem/progenitor cell accumulation in brain vessels, or within the brain parenchyma. Usually, only isolated cells or small clusters are seen in the brain, despite the clear benefits demonstrated in terms of brain repair or neurological status. Our finding that treatment with EPC-secreted factors enhances angiogenesis as much as EPC treatment might explain the therapeutic effects of these cells, even if they do not anchor or infiltrate into the brain parenchyma.

Importantly, and from a translational point of view, our group recently reported that human EPCs obtained from the peripheral blood of stroke patients secrete important growth factors including those identified in the present study [illegible]. Therefore the possibility to treat patients with autologous EPC-CM seems a promising therapeutic strategy to test in the near future.

In the present study, our aim was to demonstrate increased neurogenesis after EPC-based treatments, but only mild effects could be observed. The histological analysis of axonal tracts confirmed the enhancement of axonal reorganization towards cortical peri-infarct areas in animals receiving EPC cell therapy two weeks after the ischemic event. This enhancement occurred near peri-infarct areas with enhanced angiogenesis and in close proximity with other areas of neuroblast migration. The coupling between angiogenesis and neurogenesis has been described to occur endogenously in peri-infarct areas after stroke [illegible]. In fact, Ohab et al. elegantly demonstrated that these two processes are spatially linked in a neurovascular niche that is causally linked through the vascular production of growth factors such as stromal-derived factor-1 and angiopoietin-1 [3]. Our study demonstrated that EPC production of other growth factors such as VEGF, FGF-b and PDGF-BB could contribute to enhancing angiogenesis and neurogenesis in peri-infarct areas. The specific link between these two processes after EPC-based angiogenic therapy remains to be established.

Doublecortin is a 40–53 kDa microtubule binding protein required for the normal neural migration of immature neurons (neuroblasts) and is expressed in dividing neuronal precursor cells and immature neurons. Here, we noted only a mild increase in proliferating DCX-positive cells in the dorsolateral SVZ of animals treated with CM. However, we could not find a higher number of mature neurons (NeuN-positive) in animals receiving proangiogenic treatment compared to vehicle-treated animals or shams. The spatial and temporal pattern of proliferation, migration and localization of neuroblasts in our study corresponded to what other authors have described to occur endogenously in animals with cortical infarcts [illegible]. However, further studies exploring longer timepoints are needed to confirm whether or not the maturation into functional neurons occurs and the true effects of EPC-based therapies on neurogenesis.

Finally, endogenous axonal plasticity and functional recovery have been shown to be potentiated by the intracerebral transplantation of human neural progenitor cells one week after cerebral ischemia based on dendritic length, arborization, axonal sprouting and transport [illegible]. Moreover, the reparative effects of stem cell therapies seemed to be mediated by cell-secreted factors, such as VEGF [35]. In agreement with these data, our results support the importance of cell-free angiogenesis or neuroprogenitor therapies, perhaps over the reparative potential of the cells per se. To our knowledge, our study is the first to report the reparative potential of EPC-secreted factors after stroke.

Our study had some limitations. First, the number of cells administered to the animals (and the derived CM) ranged from [illegible], because primary EPC cultures yielded different amounts of cells. However, this allowed us to establish that there was no association between the number of administered cells and vessel density or neuroblast extension. Therefore, increasing the number of cells should not increase the regenerative potential, at least for the tested range of cells. However, further studies are needed to investigate if larger numbers of cells could offer a better therapeutic approach. Second, our mice showed some spontaneous functional recovery, as seen in the vehicle group, although it did not reach statistical significance. We are aware that this point limits the power of the functional outcome recovery of our proposed proangiogenic treatments. In this regard, we want to highlight that to date, several tests have been demonstrated to be useful to assess short-term neurological deterioration in mice with cortical lesions. However, no robust data have been published for long-term deterioration [illegible]. Finally, we hypothesize that the mild effects found on neuronal remodeling and neurological recovery could be improved with other therapeutic approaches, such as by intracerebral administration of the proposed pro-angiogenic treatments. However, we believe that the translation of these non-invasive therapies for stroke to humans could be shorter if given intravenously, since this route of administration could present advantages in terms of safety and feasibility.

Supporting Information

Table S1 Blood gas, electrolytes, glucose and hemoglobin measured 30 minutes after treatment during isoflurane anesthesia in 100% Oxygen.
(DOCX)

Author Contributions

Conceived and designed the experiments: AR AM MHG SLP LGB JM. Performed the experiments: AR AM MNS EMS SLP VB FB AP LGB. Analyzed the data: AR AM MHG SLP AP JM. Contributed reagents/materials/analysis tools: AM MNS VB AP FB. Wrote the paper: AR AM MNS EMS MHG SLP VB FB AP LGB JM.

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Article 2

Cerebral Ischemia and Matrix Metalloproteinase-9 Modulate the Angiogenic Function of Endothelial Progenitor Cells.

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Cerebral ischaemia and matrix metalloproteinase-9 modulate the angiogenic function of early and late outgrowth endothelial progenitor cells

Anna Morancho [a], **Mar Hernández-Guillamon** [a], **Cristina Boada** [a], **Verónica Barceló** [a], **Dolors Giralt** [a], **Laura Ortega** [a], **Joan Montaner** [a], **Anna Rosell** [a, *]

[a] *Neurovascular Research Laboratory, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain*

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Abstract

The enhancement of endogenous angiogenesis after stroke will be critical in neurorepair therapies where endothelial progenitor cells (EPCs) might be key players. Our aim was to determine the influence of cerebral ischaemia and the role of matrix metalloproteinase-9 (MMP-9) on the angiogenic function of EPCs. Permanent focal cerebral ischaemia was induced by middle cerebral artery (MCA) occlusion in MMP-9 knockout (MMP-9 KO) and wild-type (WT) mice. EPCs were obtained for cell counting after ischaemia (6 and 24 hrs) and in control animals. Matrigel assays and time-lapse imaging were conducted to monitor angiogenic function of WT and MMP9-deficient EPCs or after treatment with MMP-9 inhibitors. Focal cerebral ischaemia increased the number of early EPCs, while MMP-9 deficiency decreased their number in non-ischaemic mice and delayed their increase after ischaemia. Late outgrowth endothelial cells (OECs) from ischaemic mice shaped more vessel structures than controls, while MMP-9 deficiency reduced the angiogenic abilities of OECs to form vascular networks *in vitro*. Treatment with the MMP inhibitor GM6001 and the specific MMP-9 inhibitor I also decreased the number of vessel structures shaped by both human and mouse WT OECs, while exogenous MMP-9 could not revert the impaired angiogenic function in MMP-9/KO OECs. Finally, time-lapse imaging showed that the extension of vascular networks was influenced by cerebral ischaemia and MMP-9 deficiency early during the vascular network formation followed by a dynamic vessel remodelling. We conclude that focal cerebral ischaemia triggers the angiogenic responses of EPCs, while MMP-9 plays a key role in the formation of vascular networks by EPCs.

Keywords: endothelial progenitor cell • matrix metalloproteinase-9 • cerebral ischaemia • angiogenesis • neurorepair • stroke

Introduction

Stroke is a major cause of morbidity and mortality worldwide, but the only available treatments are reperfusion therapies using tissue plasminogen activator or clot retrieval interventions in certain patients with acute ischaemic strokes. However, only 2–5% of stroke patients are receiving thrombolytic therapy to restore the blood flow [1], and the impact of clot retrieval interventions is still under evaluation. In this context, as important as neuroprotection therapies, it is the enhancement of endogen neurorepair responses that contributes to stroke recovery [2]. It has been proposed that the activation of angiogenesis and neurogenesis in the confused peri-infarct areas might remodel the damaged frontier tissue leading to behavioural recovery [3]. For that reason, the enhancement of endogenous angiogenesis after stroke, which can be activated with cell-based therapies, is considered to be crucial in future neurorepair strategies.

Angiogenesis is defined as the growth of new blood vessels from pre-existing vascular structures and EPCs have been shown to participate in neovascularization in the adult [4]. Circulating EPCs can be mobilized endogenously in response to ischaemia, home to sites of neovascularization and differentiate into endothelial cells, becoming a key mode for endothelial generation and vessel repair [5]. No single marker exists for this population of progenitor cells and it is accepted that at least two subsets can be obtained under cell culture: early EPCs, which appear early in cultures with spindle-shaped morphology (also named circulating angiogenic cells) and OECs, which appear later and present clonogenic and tubulogenic potential [6–9]. Several

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*Correspondence to: Anna ROSELL,
Neurovascular Research Laboratory, Vall d'Hebron Research Institute,
Universitat Autònoma de Barcelona, Passeig Vall d'Hebron 119-129,
Barcelona 08035, Spain.
Tel.: +34934894029
Fax: +34934894015
E-mail: anna.rosell@vhir.org

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studies using in vitro and in vivo models have demonstrated the role of EPCs as an emerging agent in cell-based treatment in rat and/or cerebral ischaemia [10–13]. The factors influencing EPCs function are still being identified and under investigation, as their modulation might improve future cell-based therapies.

During new vessel formation, one of the earliest steps is the degradation of the basal membrane, and MMPs are key players that could determine the success of this complex process [14]. Among them, the gelatinase MMP-9 has been shown to be essential for capillary branching, invasion and tube formation of endothelial cells [15, 16]. Additionally, MMP-9 has been shown to play a dual role after ischaemia, their upregulation being detrimental in the acute phases, but becoming essential for an effective neurorepair [17–21].

Our hypothesis is that cerebral ischaemia is a trigger for EPC release and functions, while MMP-9 deficiency reduces EPC levels and impairs angiogenic function in the context of cerebral ischaemia. For this purpose, EPC cell culture yields and function were explored in MMP-9-deficient mice compared with WT animals subjected to middle cerebral artery occlusion. We demonstrate that the angiogenic responses of EPCs are enhanced by the ischaemic insult and impaired in the absence of MMP-9. To further test our hypothesis, the function of EPCs from human subjects was also studied in the presence of two MMP inhibitors, demonstrating the key role of MMPs and MMP-9 in the vasculogenic function of EPCs. Time-lapse imaging shows for the first time the patterns of vessel network formation, which are clearly aberrant in MMP-9-deficient EPCs and enhanced in ischaemia-stimulated EPCs.

Materials and methods

Animals

Age-matched male mice KO for MMP-9 (MMP-9 KO) and WT mice (strain background FVB; from Jackson Laboratories, Sacramento, CA, USA) were used in this study. Matrix metalloproteinase-9 KO mice were generated by replacing part of exon 2 and all of intron 2 with a phosphoglycerate kinase-neomycin cassette as described by Vu et al. [22]. Both mice genotypes (MMP-9−/− and MMP-9+/+) were bred in-house and the offspring was used for experimentation. Genotyping analysis was performed as detailed in supplemental data. All animals were housed in a temperature-controlled room and maintained on a 12-hr light/12-hr dark cycle. Only adult males (25–35 g) were included in the study and free access to food and water was allowed before and after surgery. A total of 92 mice were initially included for the middle cerebral artery occlusion (MCAO) model (ischaemic and sham animals). All procedures were approved by the local Animal Care Committee and were conducted in compliance with the Spanish legislation and in accordance with the Directives of the European Union.

Permanent focal cerebral ischaemia

A permanent focal model of stroke by occluding the middle cerebral artery (MCA) affecting the cortex was induced as previously described (see Supporting Information) [23]. Sham animals underwent a surgical procedure without the induction of the MCA occlusion. Six or 24 hrs after the ischaemia or after 24 hrs of the surgery in sham group, mice were killed.

Mouse endothelial progenitor cell cultures

Mouse early EPCs were obtained as previously described [24]. Spleens from WT and MMP-9-KO mice were obtained at 6 or 24 hrs after ischaemia and in sham animals after 24 hrs. A total of 36 cell cultures were performed from 36 mice (two spleens each) and images from five representative fields were taken at 200× at day 5. EPCs were defined as Ac-LDL-stained cells at day 5 as previously described and quantified [24, 25]. Cell counts were performed by an investigator blinded to the experimental groups. Subsequent cultures were cultured for further expansion. These cells, presenting cobblestone morphology, are late outgrowth endothelial cells, named OECs, and were used for in vitro vessel formation (Matrigel™ assay; see Figure S1). Detailed methods are available in Supporting Information.

Human blood EPCs cultures

Human OECs were obtained as previously described from peripheral blood from healthy controls (aged from 39 to 50) [26]; detailed methods are available in Supporting Information.

Immunocytochemistry

Standard EPC phenotyping was performed in mouse and human OECs for von Willebrand factor, KDR and CD133 antigens. Methods are available in Supporting Information.

In vitro vessel formation

To assess the role of ischaemia and MMP-9 on angiogenesis, Matrigel assay of OECs (Matrigel™ matrix; BD Biosciences, San Jose, CA, USA) was used for in vitro vessel formation as previously described [25]. Experimental groups were tested: mouse OECs obtained from ischaemic (24 hrs) or sham mice and from naive non-ischaemic control OECs, respectively, or human OECs. Additionally, mouse WT and human cells were treated with the MMP inhibitor GM6001 (CC1010, EMD Millipore, Darmstadt, Germany) at 10 or 20 µM or the specific MMP-9 inhibitor I (444278, EMD Millipore) at 100 nM for mouse or 0.5 and 1 µM for human cells. Finally, MMP-9-KO cells were treated with conditioned media (CM) obtained from WT OECs or with 20 or 40 nM recombinant mouse pro-MMP-9 (R&D systems, MN, USA) at 20 or 40 nM. Detailed methods are available in Supporting Information. The number of complete rings and the total tube length (perimeter of the complete rings) were counted by ImageJ software (NIH, Bethesda, MD, USA) by an investigator blinded to the treatment. Mean values were used for comparisons between cell types and experimental treatments with MMP inhibitors. CM or recombinant MMP-9 were also used as percentage of the non-treated group.

Cell viability

Cell viability assay was additionally performed as previously described [26] to assess the potential toxicity of the MMP inhibitors and the recombinant MMP-9 to the OECs. Detailed methods are available in Supporting methods.

Live time-lapse imaging for *in vitro* vessel formation

The formation of vessel-like structures by mouse OECs was assessed during 24 hrs using time-lapse imaging. Experimental groups were: WT control, WT ischaemic, MMP-9 KO control and MMP-9 KO ischaemic OECs. Standard Matrigel assay was conducted as described above and image acquisition started 1 hr after seeding and then every 30 min. up to 24 hrs. An Olympus inverted microscope-TIRFM cell^R (Olympus Biosystems, Tokyo, Japan) with temperature, CO2 and humidity control was used. Two images per well were acquired at 40× and 100×. Complete rings and branching points (points joining three or more connections) were counted every 2 hrs by an investigator blinded to the treatment.

Gelatin zymography

Presence of MMP-9 in CM from WT control and ischaemic cultures was confirmed as described in the supplementary data.

Brain vasculature quantification

A group of eight WT and eight MMP-9 KO mice were subjected to permanent focal cerebral ischaemia (n = 4 WT and n = 4 MMP-9 KO) or sham surgery (n = 4 WT and n = 4 MMP-9 KO). To assess endogenous angiogenesis after 21 days, mice were injected intravenously with 50 μg of DyLight 594 labelled tomato lectin (*Lycopersicon esculentum*; Vector Laboratories, Burlingame, CA, USA) and sacrificed by cardiac perfusion of 4% paraformaldehyde under deep anaesthesia. Brains were extracted and the cortical peri-infarct area analysed. Imaged vessels presented a diameter smaller than 10 μm typical of microvessel structures. Detailed methods are available in Supporting Methods.

Statistical analysis

SPSS 15.0 package was used for statistical analysis. Shapiro-Wilk test was used to verify if variables had a Gaussian distribution. Normally distributed variables are expressed as mean ± SD and compared by t-tests. Statistical significance for intergroup differences was assessed by Student's t-test or one-way ANOVA followed by Tukey post-hoc test. A P < 0.05 was considered statistically significant at a 95% confidence level. Extreme values (two outlying groups > 2SD in the 21 hrs ischaemic group) only affected early EPC counts.

Results

Characterization of MMP 9-deficient mice and EPCs

Wild-type and MMP-9 KO genotypes were confirmed by PCR (Fig. S2). A total of 32 mouse EPCs cultures were finally included in the study after discarding extreme values and contaminated cultures. Colonies of outgrowing OECs were obtained in 6 out of 17 cultures from WT mice (35.3%) and in 2 out of 15 from MMP-9 KO mice (13.3%) displaying two different morphologies: cobblestone or spindle-shape. No differences were seen regardless of mouse MMP-9 genotype in terms of cell morphology (Fig. 1A and B). Immunocytochemistry showed that both WT and MMP-9 KO mice and human OECs were positively stained for endothelial markers (vWF and KDR) and CD133 progenitor cell marker (Fig. 1C and D) regardless of morphology type (see additional immunocytochemistry in Fig. S3).

Influence of focal cerebral ischaemia and MMP-9 deficiency on EPC yields

The number of early EPCs as spindle-shaped cells in cultures was counted (Fig. 2A). Our results showed that in WT mice the number of EPCs was altered by ischaemia (Fig. 2B; P = 0.05), and although an increase was detected at 6 hrs this was not significant (P = 0.366), nor was the change detected at 21 hrs (P = 0.307). Conversely, in MMP-9 KO mice the ischaemic insult altered the complete temporal profile (Fig. 2B; P = 0.049) by delaying the peak in the number of EPCs being significantly higher 21 hrs after ischaemia compared with sham animals (P = 0.041). Matrix metalloproteinase-9 deficiency strongly decreased the number of EPCs in sham and 6 hrs ischaemic mice compared with WT mice (P = 0.012 and P = 0.019, respectively; see Figure 2C). At 21 hrs, WT and MMP-9 KO mice presented a similar number of EPC yields (P = 0.969) as a consequence of the delayed increase in EPCs after the ischaemic event in MMP-9 KO mice.

Focal cerebral ischaemia enhances the ability of WT mouse OECs to form vessel-like structures determining the maximum extension of vessel-like networks

Figure 3A shows representative images of vessel-like structures shaped by OECs of different studied groups. Quantification of Matrigel assays showed that WT ischaemic OECs were able to shape more vascular networks than sham OECs. Ischaemic cells obtained from WT mice connected a higher number of rings (P = 0.047) and shaped larger networks (P = 0.052) than control cells. In contrast, in MMP-9 KO cells the ischaemic situation only increased the number of rings (P = 0.035) but had no effect on the network length (P = 0.313; see Figure 3B and C).

Time-lapse imaging revealed that generation of vessel-like structures into a Matrigel matrix began only 2 hrs after seeding, while the maximum number of vessel-like structures was shaped approximately at 10 hrs as shown in Figure 3D and E and Figure S4. Interestingly, the construction period was followed by a dynamic remodelling seen only in the supporting video files of time-lapse imaging (Videos S1–S4). Table 1 shows significant differences on vessel network formation during the established construction period versus the remodelling period. Time-lapse imaging results confirmed that angiogenic abilities of OECs are strongly enhanced in WT ischaemic cells (P < 0.05 versus WT control cells). However, in the context of

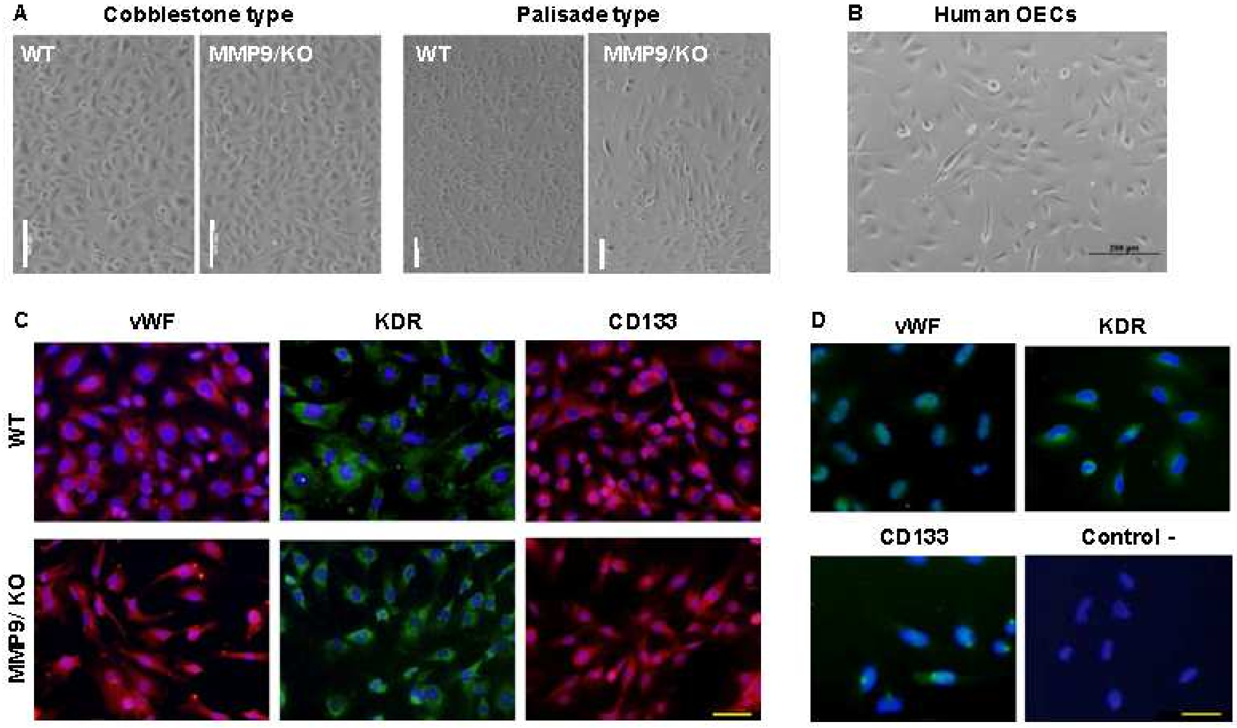

Fig. 1 Immunophenotypic characteristics of outgrowth endothelial cells (OECs) from wild-type (WT) and matrix metalloproteinase-9/knockout (MMP-9/KO) mice and human controls. (**A**) Representative images of OECs from WT and MMP-9/KO obtained from ischaemic mice showing cobblestone or palisade cell morphologies (bar = 100 μm). (**B**) Representative micrographies from human OECs. Immunofluorescent staining of OECs showing positive signal (red or green) for von Willebrand factor (vWF), KDR and CD133 (bar = 50 μm) in mouse palisade-type (**C**) and human cobblestone-type cells (**D**). The blue signal corresponds to nuclear DAPI.

MMP-9 deficiency, ischaemic cells were not superior to control cells showing the importance of MMP-9 for angiogenic responses of EPCs (Fig. 3D and E, Figure S4 and Videos S1–S4).

MMP-9 deficiency impairs the formation of vessel-like structures by mouse OECs

Control cells with MMP-9/KO genotype showed reduced angiogenic abilities when compared with WT cells as demonstrated by less rings and shorter total tube length shaped by MMP-9/KO control OECs in Matrigel™ assays ($P - 0.001$ and $P - 0.001$, respectively); see Figure 4A. Time-lapse imaging confirmed the influence of MMP-9 deficiency in the vessel network formation over time (Fig. 4B). The video images (Videos S1 and S3) also illustrate differences in the patterns of movement, showing that WT OECs can move much faster and make more cell-to-cell connections than MMP-9/KO OECs, which show clear impaired function. The same experimental groups were tested using ischaemic cells as seen in Figure 4C. Ischaemic MMP-9/KO OECs showed impaired angiogenic function compared with WT ischaemic cells by forming less rings ($P - 0.011$) and diminishing total tube length ($P - 0.016$). Figure 4D, Figure S4, Videos S2 and S4 show how the MMP-9 deficiency impairs the vessel network formation over time also in ischaemic cells, demonstrating the importance of MMP-9 for angiogenic responses of EPCs in the context of ischaemia.

To further confirm the role of MMP-9, WT control cells treated with the broad spectrum MMP inhibitor GM6001 and the specific MMP-9 inhibitor I significantly reduced their angiogenic abilities by decreasing the number of rings compared to non-treated cells ($P < 0.001$ and $P - 0.04$, respectively) and the total tube length ($P < 0.001$ and $P - 0.054$, respectively); see Figure 5A. The addition of exogenous MMP-9 by adding CM from WT OECs containing MMP-9 (Fig. S5), 20 or 40 nM of recombinant pro-MMP-9 could not revert the ability to form vessel-like structures of MMP-9/KO cells ($P > 0.05$ for ring structures and total tube length for all experimental conditions) as shown in Figure 5B. In addition, the treatment of ischaemic WT OECs with GM6001 and MMP-9 inhibitor I also reduced the angiogenic performance of ischaemic OECs, showing less number of rings ($P - 0.007$ and $P - 0.044$, respectively) and diminished total tube length ($P - 0.005$ and $P - 0.044$) than non-treated cells (Fig. 5C). In MMP-9/KO ischaemic OECs, the addition of CM from WT OECs did not improve significantly the formation of vessel-like structures as shown in Figure 5D ($P - 0.579$ for number of rings and $P - 0.253$ for total tube length).

Any of the MMP inhibitor treatments or their vehicles affected the cell viability of WT control or ischaemic OECs as shown in Figure S6A and B.

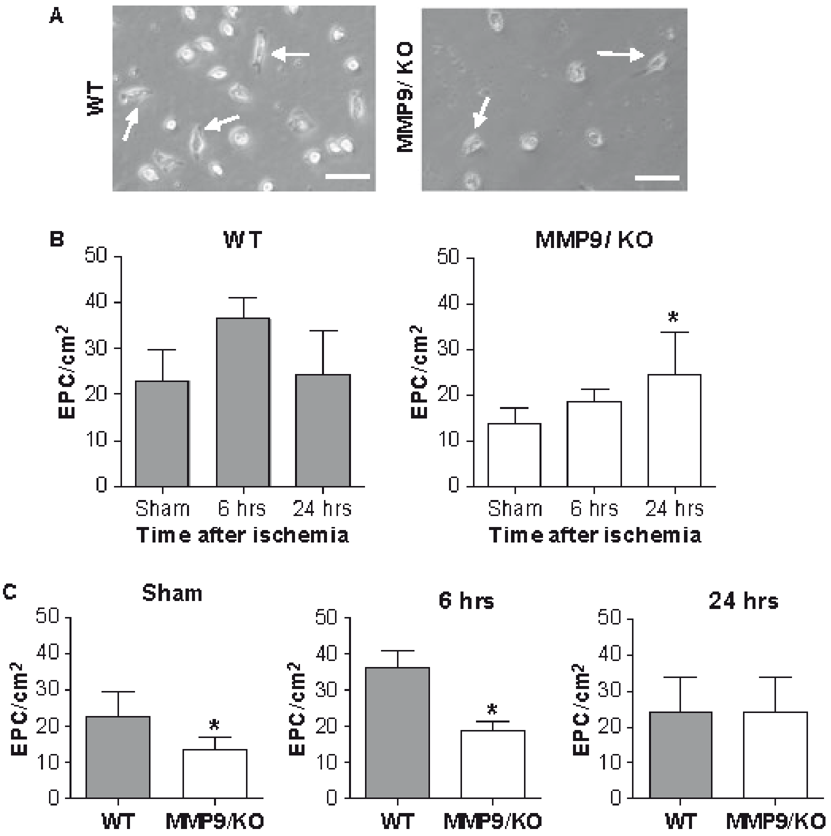

Fig. 2 Cell density of early endothelial progenitor cells (EPCs). (**A**) Representative image of spindle-shaped early EPCs (arrows); bar = 50 μm. (**B**) Bars representing cell density of EPCs from sham and ischaemic wild-type (WT) or matrix metalloproteinases-9/knockout (MMP-9/KO) mice (ANOVA followed Tukey post-hoc test; $*P < 0.05$ *versus* sham). (**C**) Number of EPCs at different time-points of ischaemia in WT and MMP-9/KO mice. (*T*-test; $*P < 0.05$). Results are expressed as mean ± SD, $n = 4$ 6/group.

Inhibition of MMPs reduces the angiogenic capacity of human OECs

Human OECs with cobblestone morphology were used in the tubulogenic assays. When treating OECs from control animals with 10 μM of GM6001, we could not observe reduced angiogenesis as seen in Figure 5F. Doubling the concentration of GM6001 to 20 μM led to a significant decrease in OECs angiogenic function by reducing both the number of vessel structures ($P < 0.001$) and total tube length ($P < 0.001$) compared with non-treated OECs; see Figure 5E and F. The specific role of MMP-9 was also demonstrated as treatment with 0.5 and 1 μM of MMP-9 inhibitor I also decreased the number of rings ($P < 0.001$ and $P < 0.001$, respectively) and the total tube length ($P < 0.001$ and $P < 0.001$, respectively).

None of the tested doses of GM6001 or MMP-9 inhibitor I and none of their vehicles showed any effect on cell viability; see Figure S6C.

In vivo angiogenesis after focal cerebral ischaemia is impaired in MMP 9-deficient mice

In a pilot study, we explored how MMP-9 deficiency could affect endogenous angio-vasculogenesis in the context of cerebral ischaemia. When comparing the amount of functionally perfused blood microvessels (as stained by lectin intravenous perfusion) in the peri-infarct cortex between control and ischaemic animals, WT mice showed an increase in the vessel density 21 days after ischaemia in some areas of the cortex affected for the infarct (P – 0.026 for posterior areas). However, in MMP-9/KO mice, there was no increase in the vessel density after the ischaemic insult in any of the studied areas ($P > 0.05$); see Figure S7.

Discussion

Our study shows that focal cerebral ischaemia promotes an acute mobilization of EPCs, while MMP-9 deficiency decreases the number of circulating EPCs delaying their mobilization after an ischaemic insult. We also demonstrate that focal cerebral ischaemia enhances the angiogenic abilities of late outgrowth EPCs *in vitro*, being partially impaired in MMP 9-deficient cells. Finally, the pharmacological inhibition of MMPs, specifically MMP-9, impaired the angiogenic function of OECs, suggesting that MMP-9 is necessary for *de novo* vascular network formation. We also suggest that endogenous angiogenesis in peri-infarct areas after focal cerebral ischaemia is partly inhibited in MMP-9/KO mice. All these data proposes MMP-9 as a key protease in post-stroke angiogenesis mediated by EPCs.

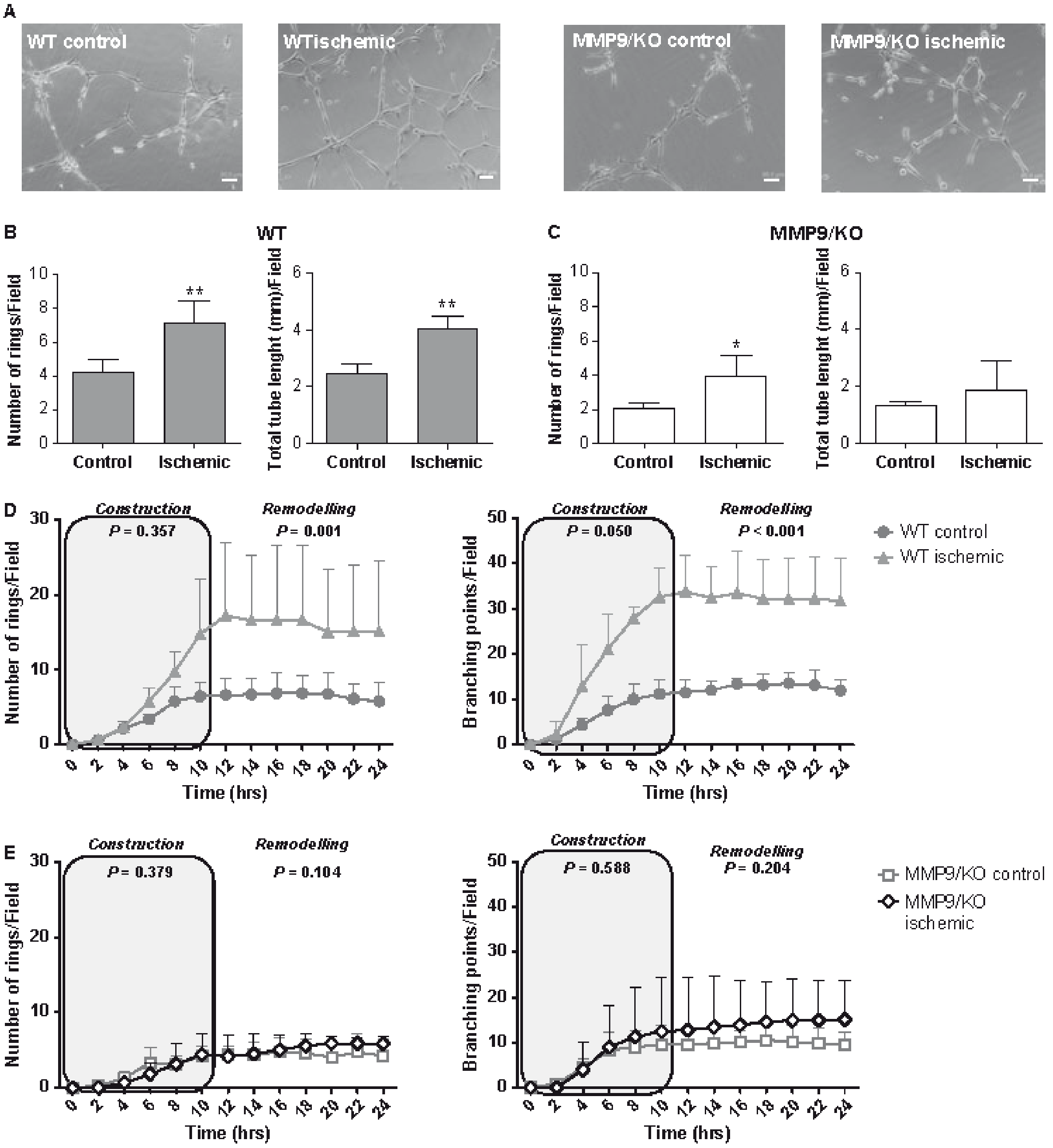

Fig. 3 Effect of ischaemia on Matrigel™ assay performance of mouse outgrowth endothelial cells (OECs). (**A**) Representative micrographs of vessel-like structures shaped by control and ischaemic (24 hrs) wild-type (WT) and matrix metalloproteinases-9/knockout (MMP-9/KO) OECs (bar = 50 μm). The number of rings and total length of vessel-like structures of control and ischaemic OECs was quantified for WT (**B**) and MMP-9/KO cells (**C**). Quantification of time-lapse imaging assay of mouse OECs on Matrigel™ matrix shows the effect of ischaemia on WT (**D**) and MMP-9/KO (**E**) on the formation and dynamics of vessel-like structures in each experimental group. Two clear patterns were observed in all groups highlighted as construction or remodelling. Results are expressed as mean ± SD; (*T*-test; $*P < 0.05$, $**P < 0.01$ *versus* control group, n = 3 4 independent experiments).

Two different types of EPCs have been described and identified in culture, early EPCs and late OECs, which present differences in phenotype, proliferation capacity and function [7]. Recruitment and mobilization of EPCs play an important role in neovascularization after tissue injury. Different studies in stroke patients have shown an increase in circulating EPCs at different time-points after the ischaemic

Table 1 Quantification of tube-like structures per hour in the construction (0–10 hrs) and remodelling (10–24 hrs) phases in Matrigel™ assays

Group	Increase in rings/hour			Increase in branching points/hour		
	0–10 hrs	10–24 hrs	*P*-value	0–10 hrs	10–24 hrs	*P*-value
Control WT	0.64 ± 0.5	−0.05 ± 0.5	<0.001	1.12 ± 0.7	0.06 ± 0.7	<0.001
Control MMP-9/KO	0.43 ± 0.5	0.00 ± 0.3	0.005	0.95 ± 1.0	0.00 ± 0.47	0.004
Ischaemic WT	1.48 ± 1.6	0.00 ± 0.9	0.005	3.27 ± 3.1	−0.70 ± 0.8	0.001
Ischaemic MMP-9/KO	0.44 ± 0.4	0.10 ± 0.3	0.005	1.24 ± 1.7	0.19 ± 0.4	0.031

Data are given as increase *per* hour and expressed as mean ± SD.

insult, while other authors have described lower levels of EPCs in stroke patients than in controls [25, 27–29]. To evaluate these contradictory data, we aimed, for the first time, to assess the mobilization of EPCs after an ischaemic insult in a mouse model of stroke. We have quantified early EPCs in short-term cell cultures from mouse spleens by counting spindle-shaped cells as previously described by other authors [24, 25]. The results show an increase in the levels of circulating EPCs early after the ischaemic insult. We have also demonstrated that MMP-9 deficiency delayed the ischaemia-induced EPC mobilization. In this sense, it is known that MMP-9 is required for the mobilization of stem and progenitor cells from the bone marrow [30, 31]. According to these studies, we have observed decreased levels of early EPCs in MMP-9/KO sham mice compared with WT animals, confirming an impaired mobilization in MMP 9-deficient animals. However, as we are considering cultured cells, we have to acknowledge that other factors could be influencing the final counts such as the cell adherence or survival capacities between WT and MMP-9/KO EPCs. This result is in agreement with other findings describing impaired mobilization of bone marrow-derived EPCs in MMP 9-deficient mice [30]. In our study, the ischaemic insult restored the impaired EPC mobilization of MMP-9/KO sham animals by increasing EPCs up to the same level as WT mice at 24 hrs. This might suggest that our model of focal cerebral ischaemia activates other triggers for bone-marrow cell mobilization in the absence of this protease or compensatory effects by other MMPs explaining the delayed response to the ischaemic trigger. Our study focuses on the mobilization and function of EPCs during the acute phase of cerebral ischaemia. However, the long-term effects and capacity of these cells to modulate neurorepair in these animal models were not in the scope of the present study and will be investigated in future studies.

In the present study, we could obtain late EPCs from spleen cultures of WT and MMP-9/KO mice, but cultures from MMP 9-deficient animals yielded a lower percentage of outgrowth cells. In this regard, other authors have reported reduced colony forming units of human circulating cells in the presence of an MMP-9 inhibitor [32]. As less cell density was found in MMP-9/KO cultures, we can hypothesize that it could influence the reduced yields of outgrowth EPCs as described by other authors [33]. Another explanation could be in the nature of MMP-9 deficiency and altered mechanisms of EPCs maturation into an endothelial phenotype. The identification of the molecular mechanisms leading to OECs appearance will provide more information of the MMP-9 role in that process.

Our results demonstrate that the OECs obtained from WT ischaemic mice showed enhanced endothelial function by performing higher number of vessel-like structures than OECs from non-ischaemic mice. On the other hand, this angiogenic function was only partially enhanced in ischaemic MMP-9/KO cells, suggesting a mild effect of the ischaemic trigger on the MMP 9-deficient OECs. The videos supplied as supporting files clearly show the persistent impairment of MMP-9/KO cells to shape vascular networks despite the ischaemic trigger. This strongly indicates that focal cerebral ischaemia can improve angiogenic abilities of OECs and preserve these abilities after maturation into endothelial-like cells, but this trigger is dependent on MMP-9 activity. To our knowledge, this is the first work comparing OECs' vasculogenic abilities from ischaemic and non-ischaemic animals *in vitro*. Supporting our data, a recent investigation has published that hypoxia pre-conditioned bone marrow mesenchymal stem cells (BMSCs) transplanted in rats after cerebral ischaemia improved the regenerative potential of standard BMSCs, suggesting mechanisms involving enhanced expression and release of trophic/growth factors [34]. The factors that cerebral ischaemia specifically activates in circulating EPCs to enhance their angiogenic potential and how the activation is maintained need to be further studied.

We have shown that MMP-9 deficiency and pharmacological inhibition of MMP-9 clearly impair the angio-vasculogenic abilities of OECs and that the addition of exogenous MMP-9 could not reverse it, suggesting that endogenous secreted MMP-9 is essential for a correct EPC function in terms of *de novo* vessel formation. We have further demonstrated that OECs from MMP-9/KO mice built aberrant vascular networks compared with WT cells, suggesting a key role of this MMP in *de novo* vascular formation. Other authors have shown similar results when inhibiting MMP-9 in human control OECs [32]. However, we show, for the first time, by time-lapse imaging, the tube formation patterns over time and specific inability of MMP-9/KO EPCS for cell migration and for the establishment of cell-to-cell interactions.

Importantly, we have successfully translated our results into the human setting. Outgrowth endothelial cells from human controls diminished their angiogenic abilities and shaped smaller vascular networks when treated with the broad spectrum MMP inhibitor or with a specific MMP-9 inhibitor.

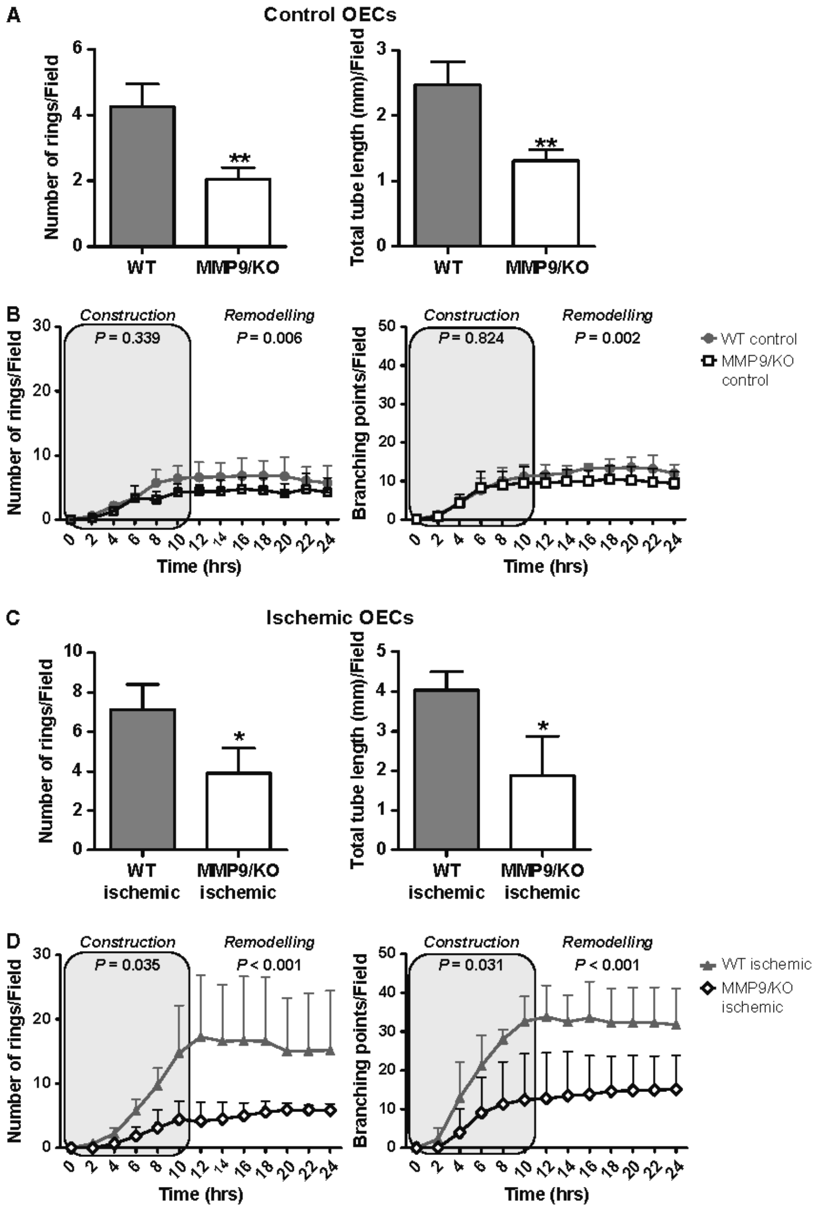

Fig. 4 Effect of matrix metalloproteinase-9 (MMP-9) deficiency on Matrigel™ assay performance of mouse outgrowth endothelial cells (OECs). Bar graphs showing *in vitro* tubulogenesis performance of wild-type (WT) control compared with MMP-9/knockout (MMP-9/KO) cells (**A**) and the corresponding time-lapse imaging (**B**) and WT and the MMP-9/KO ischaemic OECs (**C**) and the corresponding time-lapse imaging (**D**). Results are expressed as mean ± SD. (*T*-test; $*P < 0.05$, $**P < 0.01$; $n = 3\ 5$ independent experiments).

The function of MMP-9 in vascularization has been previously studied in other diseases. Johnson and colleagues described in a model of hindlimb ischaemia that MMP-9 was necessary for ischaemia-induced angiogenesis as an inhibition of tissue angiogenesis and a decreased capillary perfusion were found in MMP-9/KO mice [15]. Conversely, in myocardial infarction, a stimulation of angiogenesis has been described in MMP-9/KO animals, suggesting a possible dual role of this MMP depending on the tissue [35]. However, in the brain, MMP-9 seems to be essential for an effective neurorepair [21]. It is well described that endogenous activation of angiogenesis occurs within the first hours after cerebral ischaemia [36]. Our results support the critical role of MMP-9 in spontaneous angiogenesis after cerebral ischaemia as we have shown an increase in specific peri-infarct vessels in ischaemic WT mice, but not in MMP-9/KO animals when compared with non-ischaemic animals. It is important to highlight that with our perfusion technique of intravenous lectin to stain the blood vessels, we are only considering functional vessels with normal blood perfusion excluding any non-functional aberrant vasculature. Whether the impaired angiogenic function of OECs lacking MMP-9 observed in the present study could influence neurorecovery needs to be elucidated and warrants further studies using *in vivo* models of ischaemia receiving cellular therapy.

Finally, time-lapse imaging technique performed to examine the dynamics of vessel formation in Matrigel™ matrix confirmed that both

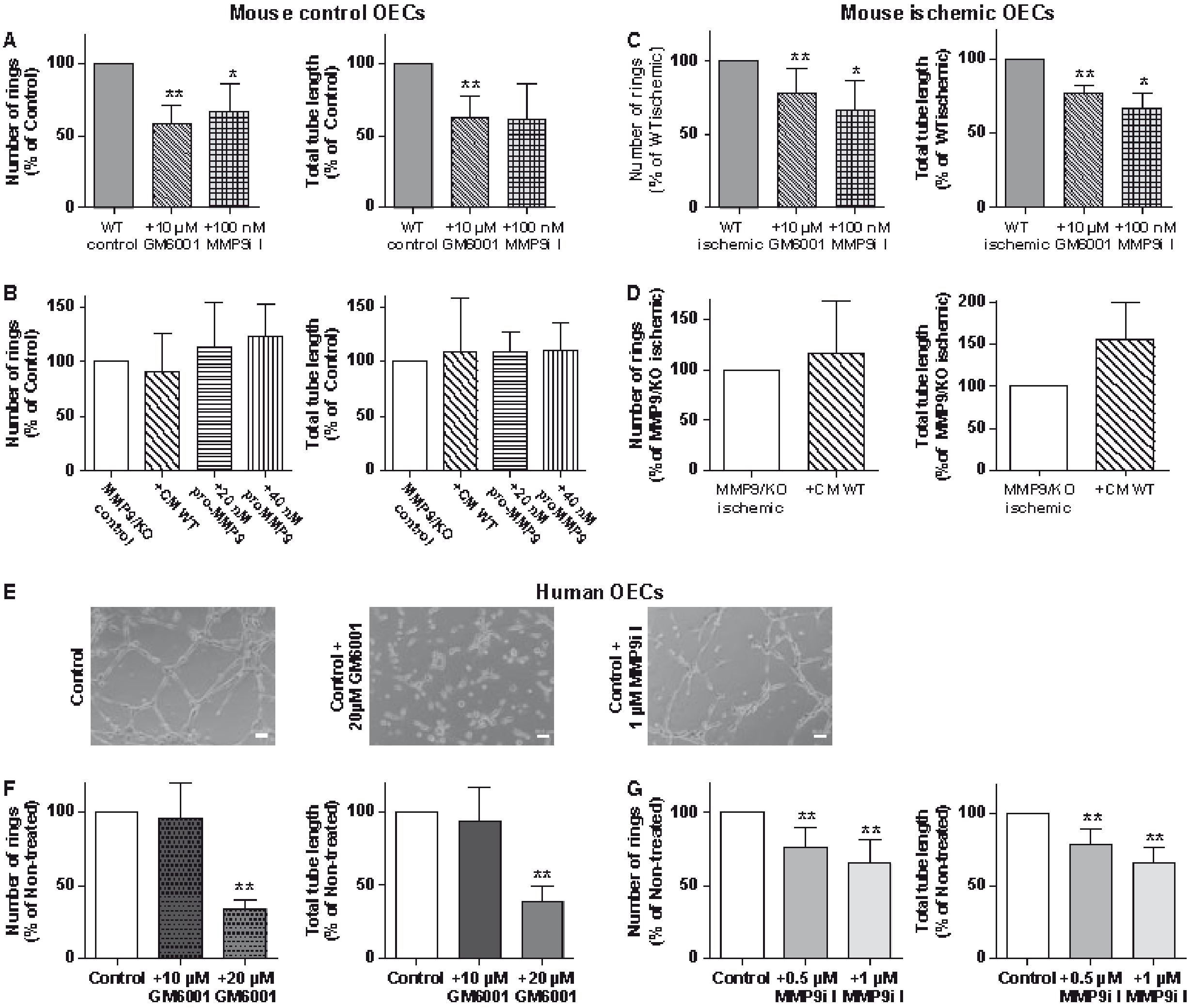

Fig. 5 Effect of matrix metalloproteinases (MMP) inhibition on Matrigel™ assay performance of mouse and human outgrowth endothelial cells (OECs). (**A**) Bar graphs showing impaired angiogenic function of wild-type (WT) control cells when treated with GM6001 and MMP-9 inhibitor I (MMP-9i I). (**B**) Bar graphs showing that angiogenic performance could not be reverted when MMP-9/knockout (MMP-9/KO) OECs were treated with exogenous MMP-9 contained in conditioned media (CM) of WT OECs or by recombinant pro-MMP-9 treatment. (**C**) Bar graphs showing the effect of pharmacological MMP inhibition of the angiogenic function of WT ischaemic cells. (**D**) The addition of CM of ischaemic WT OECS could not significantly revert the angiogenic impairment of MMP-9/KO ischaemic cells. (**E**) Representative micrographs of vessel-like structures from human OECs in the presence or absence of GM6001 and MMP-9 inhibitor I (MMP-9i I); bar = 50 μm. Bar graphs represent number of rings and the total tube length determined in OECs treated with GM6001 at a final concentration of 10 or 20 μM (**F**) or treated with 0.5 and 1 μM of MMP-9i-I (**G**). Results are expressed as mean ± SD and as a percentage of the non-treated group; for different treatments a *T*-test *versus* the non-treated control group was performed in A–D while ANOVA followed by Tukey post-hoc test was performed for dose-response analysis in F and G; $*P < 0.05$, $**P < 0.01$; $n = 5–10$ independent experiments.

cerebral ischaemia and MMP-9 determined the extension of the vascular network. Surprisingly, our results show, for the first time, that the construction of vessel-like structures in a Matrigel™ matrix by OECs occurs only during the first hours of the assay, regardless of the presence or not of MMP-9 or a stimulus such as ischaemia, which could not accelerate or slow down the establishment of the network. After this construction phase, the cells were seen in a continuous and dynamic movement in the remodelling phase maintaining the network extension in all experimental conditions. We hypothesize that this observation may respond to the incapacity for stabilization and maturation of newly formed vessels because of the absence of supporting cells as pericytes as described [37]. Our results support additional studies to evaluate if the stability of endothelial cells in more mature vascular structures could change the pattern of vascular network remodelling.

In summary, the present study demonstrates that local cerebral ischaemia triggers the angiogenic responses of EPCs in a mouse model of stroke. Our findings indicate that MMP-9 deficiency impairs the mobilization and angiogenic abilities of EPCs, demonstrating that this protease is necessary for *de novo* vascular network formation in the context of ischaemia. We conclude that MMP-9 modulation may be important for EPC-based therapies to potentiate angiogenesis in the context of ischaemia. Further *in vivo* studies are needed to determine the role of the MMP-9 in angiogenesis in a multiple cell environment and how this metalloproteinase could be modulated, for example, generating cells that overexpress this key modulator of EPCs function.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Method for culturing EPCs: Mononuclear cells (MNC) were isolated from spleen (mouse) or blood (human) by Ficoll gradient density and seeded in fibronectin (FN) coated plates with EGM-2 medium.

Figure S2. Genotype characterization of wild-type (WT) and knockout (MMP-9/KO) mice.

Figure S3. Immunocytochemistry of cobblestone-type mouse OECs.

Figure S4. Representative microscopy images of time-lapse imaging assay of mouse OECs on Matrigel™ matrix at different time-points.

Figure S5. Gelatin zymography of conditioned media (CM) detected gelatinolytic activity of pro-MMP-9 in media from WT sham and ischaemic EPC.

Figure S6. Cell viability determined by MTT assay after MMP inhibitors or vehicle treatment.

Figure S7. Brain vasculature in WT and MMP-9/KO mice after cerebral ischaemia.

Video S1. Twenty-four hours of time-lapse imaging Matrigel™ assay of mouse WT control OECs (100×).

Video S2. Twenty-four hours of time-lapse imaging Matrigel™ assay of mouse WT ischaemic OECs (100×).

Video S3. Twenty-four hours of time-lapse imaging Matrigel™ assay of mouse MMP-9/KO control OECs (100×).

Video S4. Twenty-four hours of time-lapse imaging Matrigel™ assay of mouse MMP-9/KO ischaemic OECs (100×).

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SUPPORTING INFORMATION

SUPPORTING METHODS

Genotyping analysis

To confirm that MMP9/KO mice were homozygous null for MMP9, genomic DNA was isolated from tail samples of selected mice (n=9 MMP9/KO and n=7 WT), and the disruption of the MMP9 gene was confirmed via polymerase chain reaction (PCR). For wild-type mice we used a sense oligonucleotide primer (5'-GTGGGACCATCATAACATCACA -3') and an antisense oligonucleotide primer (5'- CTCGCGGCAAGTCTTCAGAGTA -3'). For the MMP9/KO mice a neomycin cassette was used as a sense oligonucleotide primer (5´-CTGAATGAACTGCAGGACGA -3´) and an antisense primer (5´-ATACTTTCTCGGCAGGAGCA -3´). Analysis of PCR-amplified products was performed in 1.4% agarose gel in TBE buffer followed by ethidium bromide staining of DNA bands. Five µl of the PCR-reaction product plus 1µl of loading buffer were loaded in the agarose gel and the electrophoresis was run for 20 min at 120 V.

Permanent focal cerebral ischemia

All animals were anesthetized with isofluorane via facemask (4% for induction, 2% for maintenance in air, 79%N_2:21%O_2; Abbot Laboratories, Spain) and body temperature was maintained at 36.5–37℃ using a self-regulating heating blanket and a rectal probe. Mice eyes were protected from corneal damages during surgery using an ophthalmic lubricating ointment (Lipolac^{TM}, Angelini Farmaceutica, Spain). An incision was made between the left eye and ear under an operating microscope (Leica MS5, Switzerland) and the temporal muscle was cut and divided exposing the left lateral aspect of the skull. The MCA was identified through the semi-translucent skull and a small burr hole (1-2 mm diameter) was made using a high-speed microdrill at the level of the inferior cerebral vein to expose the M1 portion, leaving the dura intact. Saline was applied to the area throughout the procedure to prevent heat injury. Cerebral blood flow (CBF) was measured continuously by laser-Doppler flowmetry using a flexible fiberoptic (0.5 mm diameter; Moor Instruments, UK) placed directly on the top of the parietal branch of the M1 bifurcation beginning 5 min before MCAO, during and after the electro-cauterization. Using a micromanipulator holding a 30G needle (0.4mm diameter) the MCA was compressed and a decrease in the CBF was ensured. Therefore, using a small vessel cauterizer (Change-A-Tip^{TM}, Aaron Medical, USA) the MCA was permanently occluded by indirect electrocoagulation thorough the 30G needle. Then, the muscle was replaced and the skin was sutured using 5-0 silk suture and magnesic metamizol (400mg/kg) was administered subcutaneously right after the procedure as analgesic. The duration of anesthesia in all animals was less than 30 min. Only mice that showed decreased CBF below 75% from baseline were used for further experiments.

Sham animals underwent all surgical procedures with the exception of the MCA occlusion (MCAO). Six, 24 or 72 hours after the ischemia, or after 24 hours of the surgery in sham group, mice were sacrificed.

Mouse Spleen Endothelial Progenitor Cell Cultures

Mouse early EPCs enriched population was obtained as described. Spleens from WT and MMP9/KO mice were obtained at 6, 24 or 72 hours after ischemia and in sham animals after 24h. A pool of 2 spleens was used for each cell culture. Spleens were mechanically minced, placed at 37ºC for 15 minutes in a 1mM EDTA solution and run thorough a 40-mm nylon membrane to obtain a cell suspension. Mononuclear cells (MNCs) were obtained by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Sweden), shortly washed with red blood cells lysis solution (150 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$ and 0.1 mmol/L EDTA in distilled water) and gently washed with complete endothelial growth medium-2 (EGM-2; Clonetics®, CA, USA), which is composed of endothelial cell basal medium (EBM) containing 20% fetal bovine serum (FBS), human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (hFGF-B), insulin like growth factor 1 (R3-IGF-1), GA-1000 (gentamicin and amphoterecin-B), heparin, hydrocortisone and ascorbic acid. Isolated MNCs were finally resuspended in EGM-2 and 10^7 MNCs were seeded on fibronectin-coated 12-well cell culture plates (4 replicates per sample) and incubated in 5% CO_2 at 37ºC. Under daily observation, first media change was performed 2 days after plating and, thereafter, media was changed every 2/3 days. Expanding cell-colonies appeared between days 10 to 20. This method has been previously described in other studies to study EPCs counts and allowed us to maintain the cells in culture to further obtain outgrowth endothelial cells whereas other techniques such as flow cytometry do not allow maintaining the EPCs in culture [1-3].

A total of 52 cell cultures were performed from 104 mice (2 spleens each) and images from five representative fields were taken at 200x on day 5.

Human Blood Endothelial Progenitor Cells Cultures

Twenty ml of blood were diluted (1:1) with PBS containing 2% FBS. Afterwards MNCs were obtained by Ficoll gradient as described for mouse EPCs, cells seeded in fibronectin-coated plates and grown to obtain OECs. All procedures were approved by the ethics committee of our institution and were conducted in accordance with the Declaration of Helsinki.

Immunocytochemistry

Standard EPC phenotyping was performed in palisade-type OECs from WT and MMP9/KO mice and cobblestone-type human OECs for von Willebrand factor, KDR and CD133 antigens. Cells were fixed with cold 4% paraformaldehyde for 20 min at room temperature (RT) and washed with PBS. Afterwards, cells were permeabilized with 0.3% triton X-100 for 5 minutes, blocking buffer (1% BSA and 5% goat serum) applied for 1 hour and incubated with primary antibodies rabbit anti-von Willebrand factor (1:100, Sigma-Aldrich, USA), mouse anti-KDR/VEGFreceptor 2 (1:100, Sigma-Aldrich, USA) and rabbit anti-CD133 (1:50, Santa Cruz, USA) overnight at 4ºC. Goat anti-rabbit Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen, USA) were used as secondary antibodies at RT for 1 hour. Samples were finally mounted in Vectashield^{TM} with DAPI (Vector Labs, USA) to counterstain cell nuclei. Negative controls received identical treatment except for the primary antibody.

In vitro vessel formation

All experiments were performed with OECs between passages 4 to 8 for mouse cells and between 7 and 12 for human cells. Briefly, 24 well-plates were coated with 200 µl cold Matrigel^{TM} growth factor-reduced basement membrane matrix and allowed to solidify at 37ºC for 30 min. Afterwards, $4x10^4$ cells per well for mouse and at $6x10^4$ cells per well for human cells seeded into Matrigel^{TM}-coated wells in basal media (without factors and FBS) or basal media plus treatment as detailed above. For MMP9/KO cells treated with CM from WT cells, EBM-2 was replaced for CM (obtained after 24 hours incubation in EBM-2) from the same experiment. Cells were incubated at 37ºC during 24 hours. Each assay was performed in duplicate and the number of complete rings (circular vessel-like structures) and the total tube length (perimeter of the complete rings) were counted by ImageJ software (NIH, MD, USA) by an investigator blinded to the treatment in 6 representative fields (100x) per well. Mean values were used for comparisons between cell types while experimental treatments with MMP inhibitors, CM or recombinant MMP9 were expressed as percentage of the non-treated group.

Cell Viability

Cell viability assay was performed to asses the toxicity of the treatments applied to to the OECs. Measurement of the reduction of 3-(4,5-dimethylthiazol- 2-yl)2,5-diphenyl-tetrazolium bromide (MTT) to produce a dark blue formazan product was performed to assess the integrity of mitochondrial function as a measure of cell viability. Briefly, $4x10^4$ OECs were seeded on fibronectin-coated 24-well plates in EGM-2 medium. Twenty-four hours later the cells were washed with PBS and treated with EBM (basal medium) plus pharmacological treatment to inhibit MMPs: the broad spectrum GM6001 and the specific MMP9 inhibitor I or their corresponding vehicles (in mouse cells: 2 mM Tris, 6 mM NaCl, 0.8 mM $CaCl_2$ for GM6001 and 14.6 mM DMSO for MMP9 inhibitor I; and in human cells: 4 mM Tris, 12 mM NaCl, 1.6 mM $CaCl_2$ for GM6001 and 146 mM DMSO for MMP9 inhibitor I) added to the same final concentration of the Matrigel^{TM} assay. The reduction of MTT was measured after 24 hours of treatment. Absorbance was measured at 590 nm and each sample was measured per duplicate to obtain a mean value and results for treatments are expressed as a percentage of the control non-treated group.

Gelatin Zymography

Conditioned mediums of WT control and ischemic EPC cultures were collected and 0.5 ml were concentrated with 10K membrane centrifugal filters (Amicon Ultra-0.5ml 10K Ultracel, Millipore, Germany), obtaining from 30 to 50 µl of concentrated media. Twenty microlitres of concentrated conditioned media were loaded and separated by 10% tris-glycine gel with 0.1% gelatin as substrate, washed with renaturing buffer (Invitrogen, USA) for 90 minutes (2x45 min) and further incubated with developing buffer (Invitrogen, USA) at 37ºC for 48 hours. Finally, the gels were stained with 0.5% Comassie blue R-250 for 1 hour and then appropriately distained (3 x 20 minutes). Molecular weight markers and human MMP2 and MMP9 (Chemicon, MA, USA) recombinant proteins were used as standards.

Brain vasculature quantification

A new group of animals (8 WT and 8 MMP9/KO mice) were subjected to permanent focal cerebral ischemia (4 animals of each genotype) or sham surgery. After 21 days, mice were injected intravenously with 80 µg of Dylight 594-labeled tomato lectin (*Lycopersicon esculentum*; Vector Laboratories, CA, USA) and sacrificed by cardiac perfusion of 4% paraformaldehyde under deep anesthesia after 10 minutes. The brains were removed, post-fixed overnight in 4% PFA and cryoprotected with 30% sucrose in PBS for 24 hours. Afterwards, the brains were frozen and embedded in OCT before storage at -80ºC. Twelve-µm thick coronal sections were collected from anterior (+1 to +0.2 bregma) and posterior (-1.7 to -2.3 bregma) areas including the lateral ventricles and the hippocampus respectively. Sections were thaw at room temperature for 30 minutes, transferred to PBS for hydratation and slices were mounted in Vectashield^{TM} with DAPI to counterstain cell nuclei. Four images (100x) of the peri-infarct cortex of ischemic mice or the respective area in sham animals were taken, and the total area of lectin positive vessels was calculated with standard computer-assisted image analysis technique (Image J free software, NIH) by an investigator blinded to the treatment. Results are expressed as the mean vessel density of the 4 images in each area.

SUPPORTING REFERENCES

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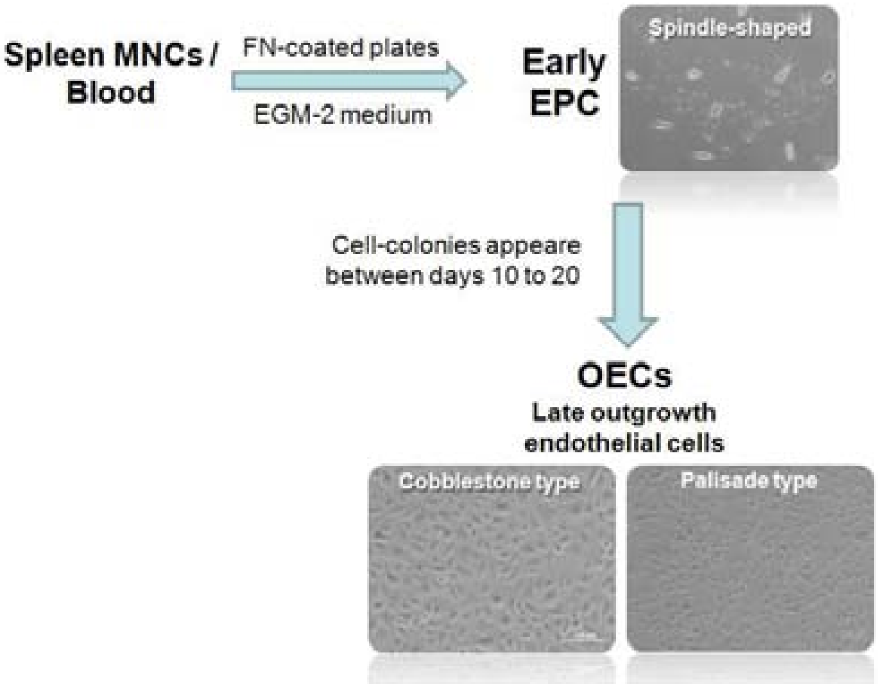
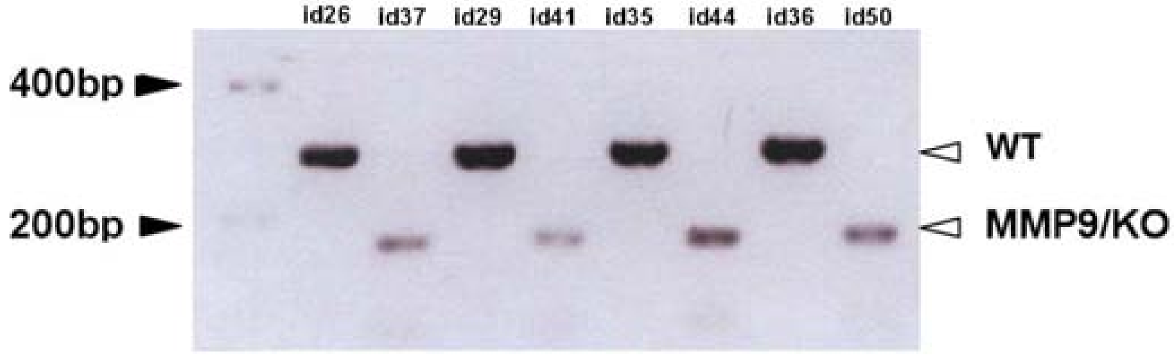
SUPPORTING FIGURES AND FIGURE LEGENDS

Supporting Figure 1.



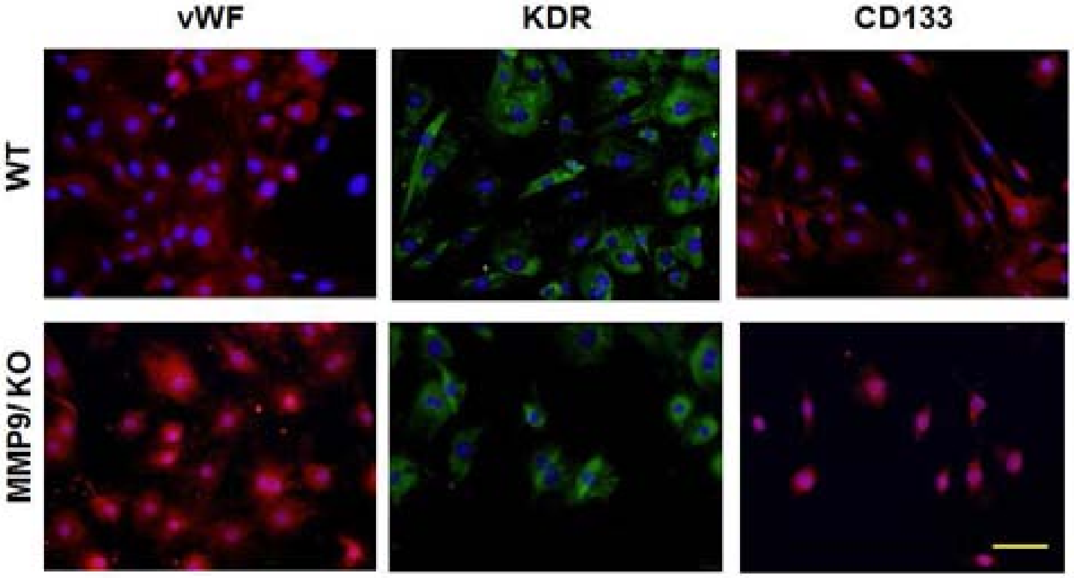
Supporting Figure 1. Method for culturing EPCs: Mononuclear cells (MNC) were isolated from spleen (mouse) or blood (human) by Ficoll gradient density and seeded in fibronectin (FN)-coated plates with EGM-2 medium. Early EPCs (also called circulating angiogenic cells, CAC) were counted as “spindle-shaped” cells. Colonies of OECs appeared as either cobblestone or palisading type.

Supporting Figure 2.



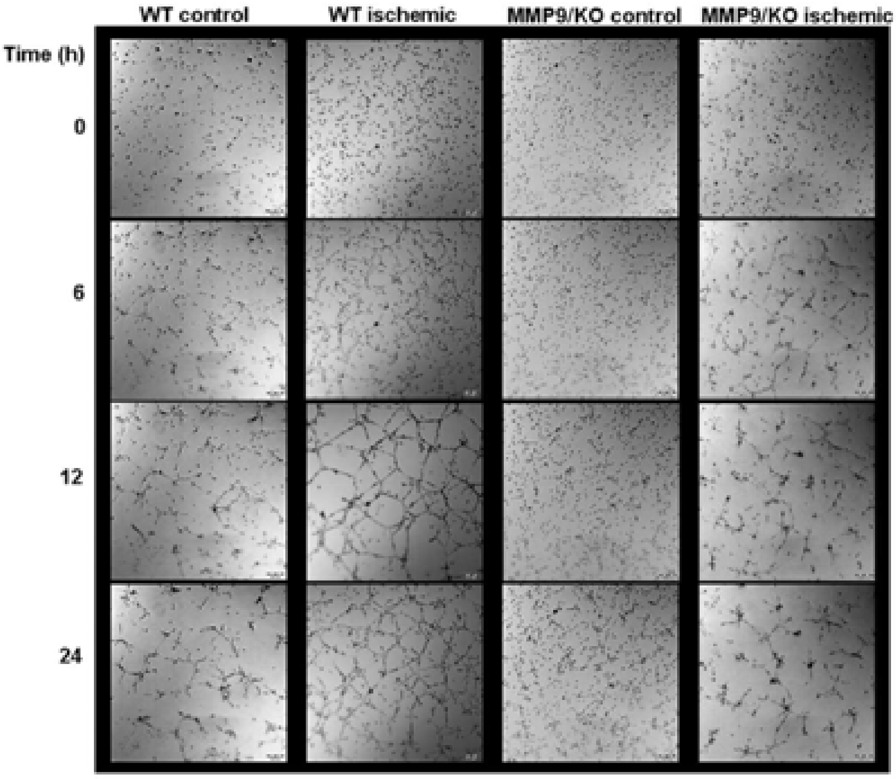
Supporting Figure 2: Genotype characterization of wild-type (WT) and knockout (MMP9/KO) mice. Agarose gel electrophoresis showing PCR products after DNA amplification of part of the MMP9 gene in WT mice (277bp band) and the disrupted MMP9 gene by neomycine resistance in KO mice (172bp band).

Supporting Figure 3.



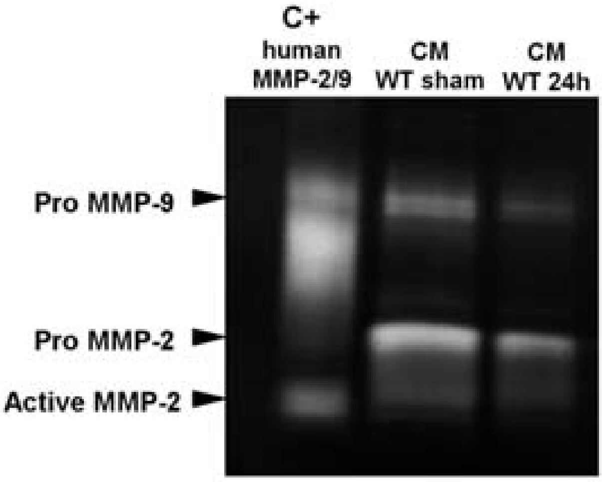
Supporting Figure 3. Immunocitochemistry of cobblestone-type mouse OECs. Immunofluorescent staining of OECs showing positive signal (red or green) for von Willebrand factor (vWF), KDR and CD133 (bar=50µm) in cells obtained from WT or MMP9/KO mouse.

Supporting Figure 4.



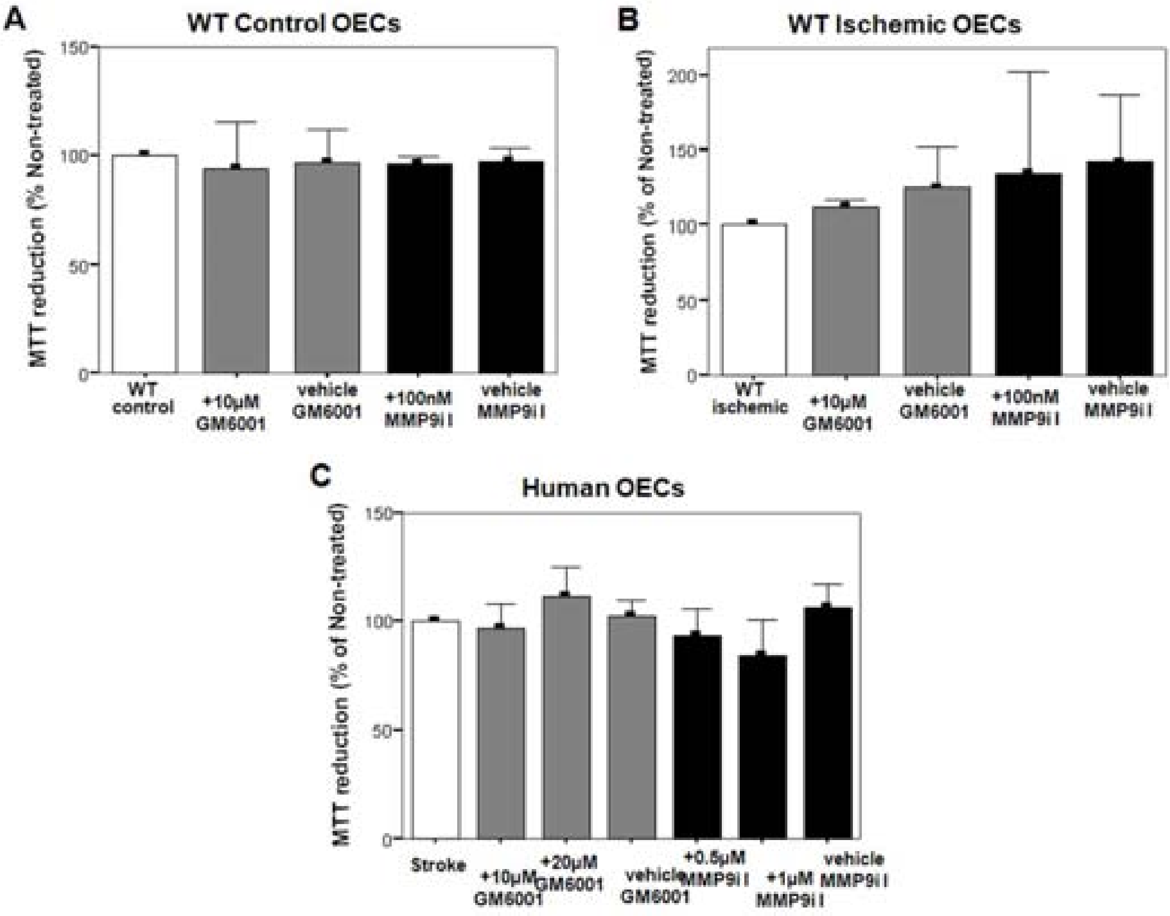
Supporting Figure 4. Representative microscopy images of time-lapse imaging assay of mouse OECs on Matrigel™ matrix at different time points. (bar=200µm).

Supporting Figure 5.



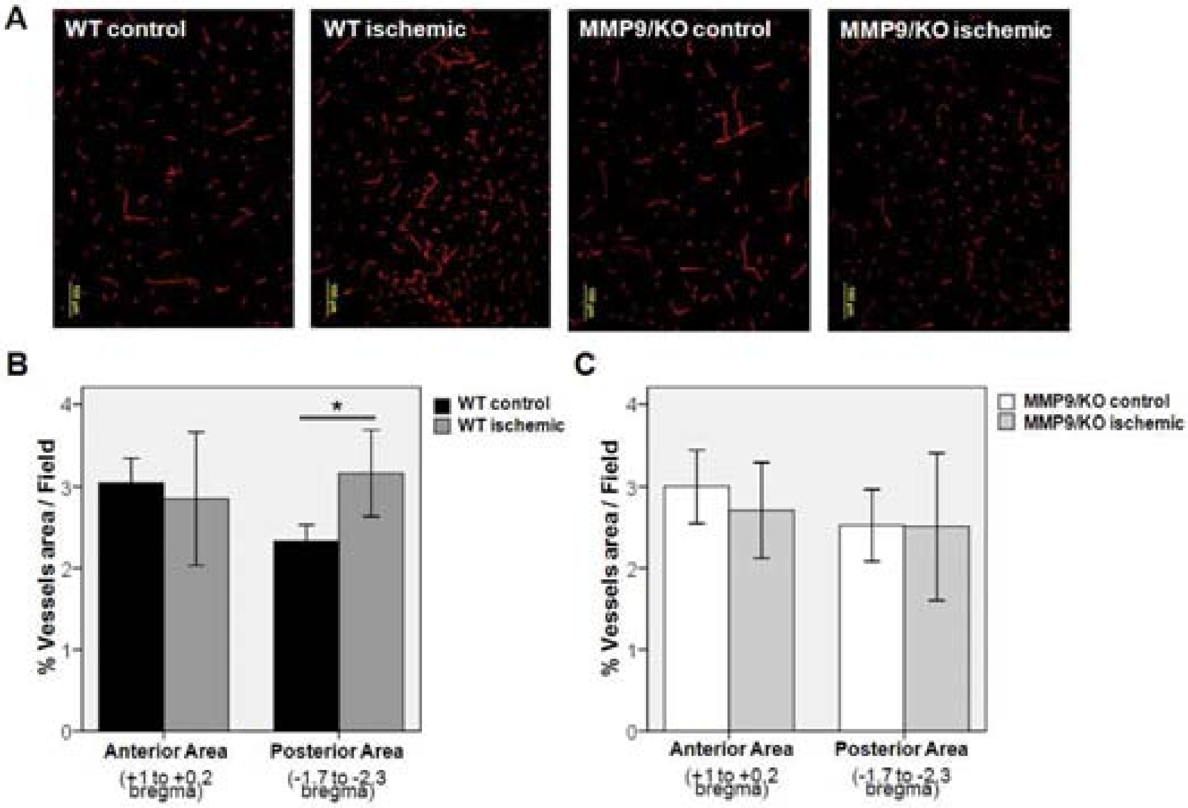
Supporting Figure 5: Gelatin zymography of conditioned media (CM) detected gelatinolytic activity of pro-MMP9 in media from WT sham and ischemic EPC.

Supporting Figure 6.



Supporting Figure 6. Cell viability determined by MTT assay after MMPs inhibitors or vehicle treatment. Bar graphs represent cell viability for OECs from WT control mouse **(A)**, WT ischemic mouse **(B)** and human control subjects **(C)**; n=4 independent experiments per group. Results are expressed as mean±SD and referred to the non-treated group. No significant differences were found.

Supporting Figure 7.



Supporting Figure 7. Brain vasculature in WT and MMP9/KO mice after cerebral ischemia. Functional blood vessels stained after lectin perfusion were quantified in the ipsilateral peri-infarct cortex 21 days after ischemia and in corresponding areas of sham mice. **A)** Representative micrographs (10X objective) of lectin staining (bar=100μm). Bar graphs show that only WT mice increased vessel density compared to non-ischemic animals in the posterior areas **(B)** while no differences were observed in MMP9/KO mice **(C)**. Data represents mean±SD, *p<0.05 (n=4 mice per group).

VIDEO FILES

Supporting Video 1: Twenty-four hours of time-lapse imaging Matrigel^{TM} assay of mouse WT control OECs (100x).

http://youtu.be/Mb4C_RR3OY0

Supporting Video 2: Twenty-four hours of time-lapse imaging Matrigel^{TM} assay of mouse WT ischemic OECs (100x).

http://youtu.be/bILFSqfB8e0

Supporting Video 3: Twenty-four hours of time-lapse imaging Matrigel^{TM} assay of mouse MMP9/KO control OECs (100x).

http://youtu.be/2r_161LRZe0

Supporting Video 4: Twenty-four hours of time-lapse imaging Matrigel^{TM} assay of mouse MMP9/KO ischemic OECs (100x).

http://youtu.be/oBp3nlRRRE8

Article 3

A new method for focal transient cerebral ischemia by distal compression of the middle cerebral artery.

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A new method for focal transient cerebral ischaemia by distal compression of the middle cerebral artery

Anna Morancho, Lidia García-Bonilla, Verónica Barceló, Dolors Giralt, Mireia Campos-Martorell, Sandra Garcia, Joan Montaner and Anna Rosell

Neurovascular Research Laboratory, Department of Neurology and Department of Internal Medicine, Universitat Autònoma de Barcelona, Vall d'Hebron Institut de Recerca, Barcelona, Spain

A. Morancho, L. García-Bonilla, V. Barceló, D. Giralt, M. Campos-Martorell, S. Garcia, J. Montaner and A. Rosell (2012) *Neuropathology and Applied Neurobiology* **38,** 617–627

A new method for focal transient cerebral ischaemia by distal compression of the middle cerebral artery

Aims: Rodent experimental models are essential for in vivo study of stroke. Our aim was to develop a reproducible method of mouse transient focal cerebral ischaemia by distal artery compression. **Methods:** The distal middle cerebral artery (dMCA) was occluded by compression with a blunted needle and cerebral blood flow was monitored by laser Doppler flowmetry to ensure appropriate occlusion and reperfusion in Balb/c mice. The ischaemic lesion was evaluated 24 h after occlusion by TTC staining and immunolabelling (NeuN, CD31, GFAP and Iba-1) while the established permanent dMCA occlusion (dMCAO) model was used as a control. The corner test was performed to evaluate neurological behaviour. **Results:** Laser Doppler flowmetry register showed a homogenous arterial occlusion among animals. Forty-five minutes of arterial occlusion did not lead brain infarction when evaluated by TTC staining 24 h after occlusion. Extending the cerebral ischaemia period to 60 min induced a cortically localized homogeneous brain infarct. No differences in infarct volume were detected between animals submitted to permanent or 60-min transient dMCAO (42.35 ± 9.88 mm^3 and 37.65 ± 12.09 mm^3 respectively). The ischaemic injury was confirmed by immunohistochemistry in the 60-min transient dMCAO model but not in the 45-min model. Neurological deficits assessed with the corner test were significant only during the first 48 h but not at long term. **Conclusions:** This work shows an easy-to-perform method for the induction of brain ischaemia and reperfusion to assess stroke repair and treatment screening, with cortically localized ischaemic cell damage, low mortality and neurological impairment in the acute phase.

Keywords: cerebral ischaemia, experimental model, middle cerebral artery occlusion, mouse, stroke

Introduction

Stroke is a major cause of morbidity and mortality worldwide [1]. Nowadays, only reperfusion therapies using tissue plasminogen activator (t-PA) are given to treat hyperacute ischaemic strokes, but only 2–5% of stroke patients are receiving thrombolytic therapy to restore the blood flow [2]. Therefore, it is desirable to develop new stroke treatments to mitigate the brain damage caused by an ischaemic episode and to reduce cellular injury or death. Reliable animal stroke models are a powerful tool to evaluate the efficacy and safety of those treatments prior to translate emerged findings into clinically stroke therapies.

Several models of cerebral ischaemia have been developed in different species, but most studies have been performed using rodent models. The occlusion of the middle cerebral artery (MCA) is the most common method to achieve focal cerebral ischaemia. The earliest animal models involved invasive techniques such as the transorbital method in nonhuman primates or the fracture and

Correspondence: Anna Rosell, Institut de Recerca Hospital Vall d'Hebron, Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain. Tel: +34 93 489 4029; Fax: +34 93 489 4015; E-mail: anna.rosell@vhir.org

removal of the zygomatic arch in rodents to approach to the proximal MCA [3,4]. Nowadays, less invasive techniques are commonly employed involving the mechanical suture, ligation or electrocoagulation of the MCA [5–9], by the injection of blood clots or the thrombotic in situ occlusion of the MCA [10–13] or by a photochemical reaction producing microthromboses after the injection of a photosensitive dye [14,15].

The optimal method of MCA occlusion (MCAO) should be technically easy to perform, inexpensive, reproducible with minimal variability of the major outcomes of interest, with low mortality rates and must cause measurable neurological deficit [16,17]. The permanent occlusion of the MCA with the distal method affecting the cortex achieves these requirements, but usually fails to accomplish well-controlled reperfusion with transient occlusions by clip or suture of the MCA [18,19] and might produce vessel damage. Transient models of ischaemia where the blood flow is restored after the ischaemic insult might represent the closest to assure the arrival of specific treatments into the injured area and to study ischaemia–reperfusion injury.

Our aim was to develop a reproducible method for transient focal cerebral ischaemia by mechanical compression of the MCA at its distal segment.

Materials and methods

Animals

Adult male Balb/c mice (25 to 30 g body weight) were obtained from Charles River Laboratories (Cerdanyola, Spain) and were given free access to food and water prior to surgery. A total of 59 animals were used for the study. All procedures were approved by the local Animal Care Committee (protocol number 16-29) and were conducted in compliance with the Spanish legislation and in accordance with the Directives of the European Union.

Model of transient cerebral ischaemia

All animals were anaesthetized with isoflurane via facemask (4% for induction, 2% for maintenance in air 79% N_2 : 21% O_2; Abbott Laboratories, Madrid, Spain). Body temperature was maintained at 36.5–37°C using a self-regulating heating blanket and a rectal probe. Mice eyes were protected from corneal damages during surgery using an ophthalmic lubricating ointment (Lipolac®; Angelini Farmacéutica, Barcelona, Spain). An incision was made between the left eye and ear under an operating microscope (Leica MS5; Leica, Heerbrugg, Switzerland) and the temporal muscle was cut and divided exposing the left lateral aspect of the skull. The MCA was identified through the semi-translucent skull, and a small burr hole (2–3 mm diameter) was made using a high speed microdrill at the level of the inferior cerebral vein to expose the M1 portion, leaving the dura intact. Saline was applied to the area throughout the procedure to prevent heat injury, keeping the area always hydrated. Regional cerebral blood flow (CBF) was measured continuously by laser Doppler flowmetry using a flexible fibreoptic (0.5-mm diameter; Moor Instruments, Devon, UK) placed on the surface of the M1 parietal branch bifurcation. CBF was recording during the entire procedure, starting 5 min before MCAO, during the occlusion and 5 min after the reperfusion. The MCA was directly compressed using a micromanipulator holding a 30 G blunted needle (0.3-mm diameter). After 45 or 60 min of occlusion (n = 8 and n = 15 respectively), the needle was carefully removed and the blood flow was restored. Arterial occlusion was considered when the CBF was maintained for 45 or 60 min (depending on the study group) below 20% of the pre-ischaemia baseline value, and reperfusion when the CBF was recovered and reached at least the 75% of baseline.

Finally, the temporal muscle was replaced, the skin was closed using 5-0 silk suture and the wound was cleaned with iodine solution. A single dose of analgesic metamizol (400 mg/kg) was administered subcutaneously right after the procedure. Total time of anaesthesia was approximately 90 min.

In a separate group (n = 4), the same surgical procedure was performed and the right femoral artery was catheterized for monitoring of blood gases (pH, pO_2 and pCO_2) 15 min before and after the ischaemia period (i-STAT CG8+ cartridges for i-STAT Portable Clinical Analyzer; Abbott Laboratories). All these animals met the criteria of arterial occlusion and reperfusion.

Model of permanent cerebral ischaemia

An established and reliable permanent focal model of ischaemia, by electrocauterization of the distal portion of the left MCA, was performed on a different group of mice (n = 7) as previously described with minor modifications [20]. The technique used was the same as the one used for induction of transient ischaemia, but on the contrary, the artery was permanently occluded using a small vessel cauterizer (Change-A-Tip™; Aaron Medical, Clearwater, FL, USA) by indirect electrocoagulation through the compressing 30G blunted needle. Only mice that showed a maintained CBF below 20% of the preischaemia during the following 5 min after artery cauterization were used for experiments. Total time of anaesthesia was less than 30 min.

Behavioural testing

The corner test was used to assess stroke severity and functional outcome [20]. Tests were performed before surgery and repeated 4 h, 24 h, 48 h, 7 days and 15 days after 60-min transient MCA occlusion. Briefly, the mouse was placed between two vertical boards (each of 30 cm × 20 cm × 1 cm) attached on one side at an angle of 30° with a small opening at the end. Mice were placed between the two boards facing to the corner and allowed to run into it, where they rear forward turning either to the left or right side. Ischaemic mice should show asymmetric turning preference. Each mouse was tested for over 20 trials and the laterality index was calculated according to the formula: laterality index = (turns to the left side – turns to the right side)/total number of turns. A laterality index of 0 is scored by animals presenting total symmetry. This test detects integrated sensorimotor function as it involves both stimulation of the vibrissae (sensory neglect) and rearing (motor response).

Infarct volume measurement

Five mice subjected to 45-min transient ischaemia, seven mice subjected to 60-min transient ischaemia and seven mice subjected to permanent ischaemia were sacrificed by cardiac perfusion of cold saline under deep anaesthesia after 24 h of distal MCAO (dMCAO). Brains were removed, sliced in one-millimetre-thick coronal sections and stained with 2.5% of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; Sigma, St Louis, MO, USA) in saline at room temperature (RT) for 20 min and ultimately fixed with 4% paraformaldehyde solution. Infarct volumes were quantified with standard computer-assisted image analysis technique (Image J free software; NIH). The infarct volume was calculated by integration of the lesion areas and considering the average of anterior and posterior views. Infarct percentage was defined as infarct volume divided by the total hemisphere volume. Oedema index was determined as the addition of all areas of the ipsilateral hemisphere divided by the addition of all areas of the contralateral hemisphere [21]. Finally, infarct volumes were corrected for oedema and results are given in cubic millimetres (mm^3).

Immunohistochemistry

Immunoreactivity for the endothelial cell marker CD31, mature neuronal marker NeuN, astroglial marker glial fibrillary acidic protein (GFAP) and microglial marker Iba-1 were examined. Four mice subjected to 45-min transient dMCAO and four mice subjected to 60-min transient dMCAO were sacrificed by cardiac perfusion of 4% paraformaldehyde (PFA) at 24 h after ischaemia. The brains were removed, post-fixed overnight in 4% PFA and cryoprotected with 30% sucrose in phosphate-buffered saline (PBS) for 24 h. Afterwards, the brains were frozen and embedded in OCT (Optimal Cutting Compound; Tissue-Tek, Torrance, CA, USA) before storage at −80°C. Twelve-micrometre-thick coronal sections were collected from anterior (+1 to +0.2 bregma) and posterior (−1.7 to −2.3 bregma) areas including the lateral ventricles and the hippocampus respectively. Sections were thaw at RT for 30 min and transferred to PBS for hydratation. Afterwards, cells were permeabilized with 0.3% Triton X-100 for 10 min, and blocking buffer (1% bovine serum albumin and 5% goat serum) was applied for 1 h. Sections were incubated with primary antibodies overnight at 4°C in blocking buffer. For immunostaining of mature neurones, anti-Neuronal Nuclei Alexa Fluor primary 488 conjugated (1:200; Millipore, Billerica, MA, USA) antibody was used. Endothelial cells were immunostained with rabbit anti-CD31 (1:20; Abcam, Cambridge, UK), microglia with rabbit anti-Iba-1 (1:500; Wako, Osaka, Japan) and astrocytes with rabbit anti-GFAP (1:200; Sigma). Goat anti-rabbit Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen, Grand Island, NY, USA) were used as secondary antibodies at RT for 1 h. Slices were finally mounted in Vectashield™ with DAPI to counterstain cell nuclei. Four images (×100) of the ischaemic cortex and four of the equivalent contralateral areas were taken, and total number of NeuN+ cells and the total area of CD31-stained vessels were calculated with standard computer-assisted image analysis technique (Image J free software). Four images (×100) of the same areas were taken for visual analysis of GFAP and Iba-1 staining.

Statistical analysis

SPSS 15.0 package was used for statistical analyses. All variables were normally distributed. Values are expressed as mean ± SD and statistical significance was assessed using the *t*-test or ANOVA followed by a Tukey *post hoc* test. Correlations between variables were evaluated using the Pearson correlation coefficient. A *P*-value less than 0.05 was considered statistically significant.

Results

Mortality

All animals included in the permanent dMCAO group and all animals subjected to 45-min transient dMCAO survived the 24-h study period. For the 60-min transient dMCAO group, three mice out of 19 (15.7%) died during surgery in the ischaemia period. The most probable cause of death seems to be the loss of spontaneous respiration under anaesthesia during the surgical procedure. After surgery reanimation, all animals included in the 60-min transient dMCAO survived the 24-h ($n = 12$) or 14-day ($n = 4$) study period.

Regional cerebral blood flow

Cerebral blood flow was monitored by laser Doppler flowmetry both in transient and permanent ischaemia, achieving a decrease >80% on CBF (considering 100% as the pre-ischaemia value) during compression of the artery or permanent occlusion by electrocoagulation (see Table 1).

The transient ischaemia was initially applied for 45 min in five mice achieving all of them the CBF established criteria for both occlusion (8.72 ± 5.1%) and reperfusion (99.66 ± 17.0%). As none of the animals presented an established infarct assessed by TTC stain, a second group of animals were submitted to 60-min transient ischaemia ($n = 16$); 15 mice achieved the CBF criteria for occlusion (12.18 ± 6.8%) and reperfusion (126.90 ± 32.2%), whereas one animal did not meet CBF criteria for reperfusion (see Table 1 and Figure 1).

Table 1. Cerebral blood flow values

Ischaemia model	*Animals (n)*	*CBF during occlusion (%)*	*CBF at reperfusion (%)*
pdMCAO	7	5.56 ± 3.2	–
tdMCAO (45 min)	9	8.72 ± 5.1	99.66 ± 17.0
tdMCAO (60 min)	15	12.18 ± 6.8	126.90 ± 32.2

Cerebral blood flow occlusion and reperfusion percentages achieved in each animal model. The CBF values (mean ± SD) are expressed in percentages considering the pre-ischaemic values as 100%.
CBF, cerebral blood flow; pdMCAO, permanent distal middle cerebral artery occlusion; tdMCAO, transient distal middle cerebral artery occlusion.

Four animals of the 60-min transient ischaemia group showed hyperaemia (>50% CBF increase of the pre-ischaemia value) upon reperfusion. This increase in the CBF had no correlation with the volume of the infarct ($P = 0.965$). Using our data is not possible to establish a relation between hyperaemia and mortality, because death occurred before reperfusion. In the same way, we can not establish a relation between hyperaemia and functional outcome as any of the animals tested in the corner test showed hyperaemia upon reperfusion.

The permanent model was tested in seven animals, and all of them achieved the CBF criteria for occlusion (5.56 ± 3.2%).

Physiological parameters

Physiological variables (pH, pCO_2 and pO_2) measured in the 60-min transient ischaemia group remained within

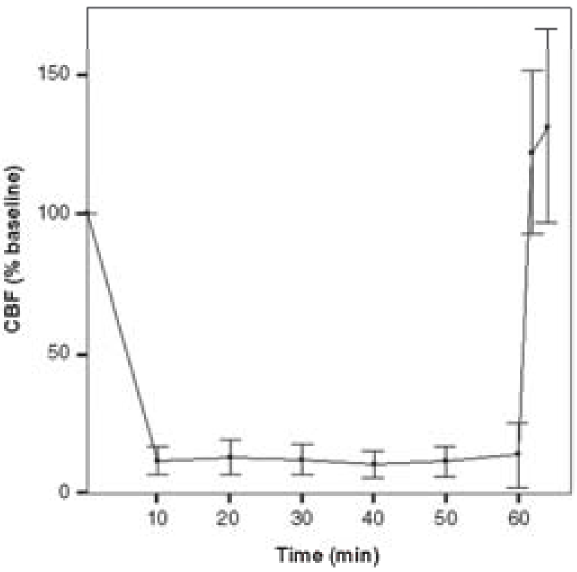

Figure 1. Representation of the cerebral blood flow (CBF) trace recorded by laser Doppler flowmetry in the 60-min transient distal middle cerebral artery occlusion (dMCAO) group (each time point represents the mean and SD of all animals, $n = 15$). CBF values were recorded continuously during the procedure and measured at 10 min (11.8 ± 5.1%), 20 min (13.1 ± 6.4%), 30 min (12.3 ± 5.3%), 40 min (10.4 ± 4.8%), 50 min (11.7 ± 5.5%), 60 min (13.9 ± 11.6%) and at the onset of reperfusion (126.9 ± 32.2%).

the normal range throughout the observation period as shown in Table 2.

Analysis of infarct volume

The TTC staining was performed to measure infarct volumes 24 h after permanent or transient ischaemia. As mentioned, no signs of infarction were seen in the five animals submitted to 45-min dMCAO (Figure 2A). On the other hand, animals submitted either to permanent dMCAO or 60-min dMCAO presented infarcts restricted to the cortical areas of the MCA territory (Figure 2B). The anatomical distribution of the infarct showed by coronal sections was similar in the transient and permanent dMCAO models (Figure 2B,C).

Sixty-minute dMCAO produced an infarct volume of 37.63 ± 12.09 mm^3, representing a 20.69 ± 7.02% of the ipsilateral hemisphere. These values were not different from mice subjected to permanent ischaemia, where the infarct volume was 42.33 ± 9.88 mm^3 ($P = 0.441$) and 22.02 ± 5.55% ($P = 0.701$) of the ipsilateral hemisphere (see Figure 2D).

Table 2. Physiological variables

	pH	*pCO$_2$ (mmHg)*	*pO$_2$ (mmHg)*
Pre-ischaemia	7.361 ± 0.03	35.13 ± 1.93	90.50 ± 7.72
Post-ischaemia	7.344 ± 0.18	37.83 ± 3.72	80.75 ± 4.35

Physiological variables measured before and after 60-min transient distal middle cerebral artery occlusion ($n = 4$). No statistically significant differences were detected between groups.

Ischaemic injury and cell death after transient distal middle cerebral artery occlusion

Tissue injury was also assessed using immunohistochemical staining to demonstrate changes affecting neurones, vessels and glial cells in the infarct area compared to the corresponding cortex area (Figure 3A).

In mice subjected to 45-min transient dMCAO, no changes were found in the number of NeuN-stained mature neurones within the anterior and posterior cerebral cortex of the ipsilateral hemisphere compared to the contralateral hemisphere (2676 ± 97 *vs.* 2668 ± 278, $P = 0.996$, and 2785 ± 345 *vs.* 2753 ± 142, $P = 0.827$ respectively). Likewise, no differences were detected when the CD31 vessel-stained area was measured in both anterior (12.02 ± 0.5% *vs.* 11.44 ± 20.7%, $P = 0.224$) and posterior (10.93 ± 0.8% *vs.* 10.69 ± 0.5%, $P = 0.631$) areas. No changes in immunoreactive GFAP-positive astrocytes were seen between the ipsilateral and contralateral cortex of these animals. Regarding Iba-1 microglial cells, an activation and mobilization of these cells that

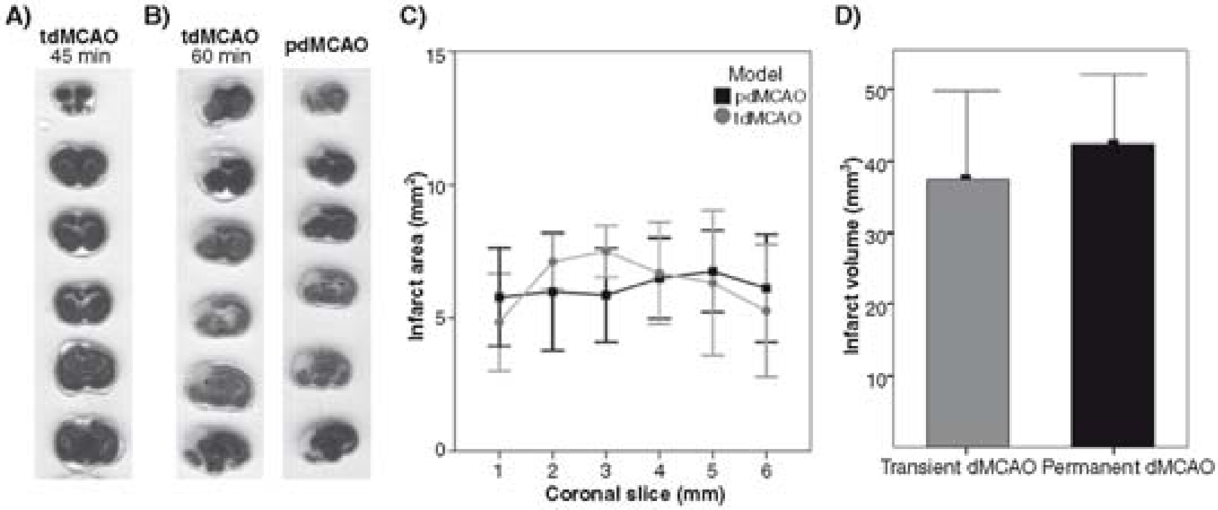

Figure 2. The TTC staining and infarct volume measurements after 45 min or 60 min of transient distal middle cerebral artery occlusion (tdMCAO) and permanent dMCAO (pdMCAO). Infarct area (in white) was not detected after 45-min transient dMCAO by TTC staining (A). Sixty-minute transient and permanent dMCAO models produced cortical infarcts as showed by TTC staining (B). Infarct area in six coronal sections from rostral to caudal (1–6 mm) (C) and infarct volume (D) were similar between the two models of ischaemia. Graphs represent mean ± SD.

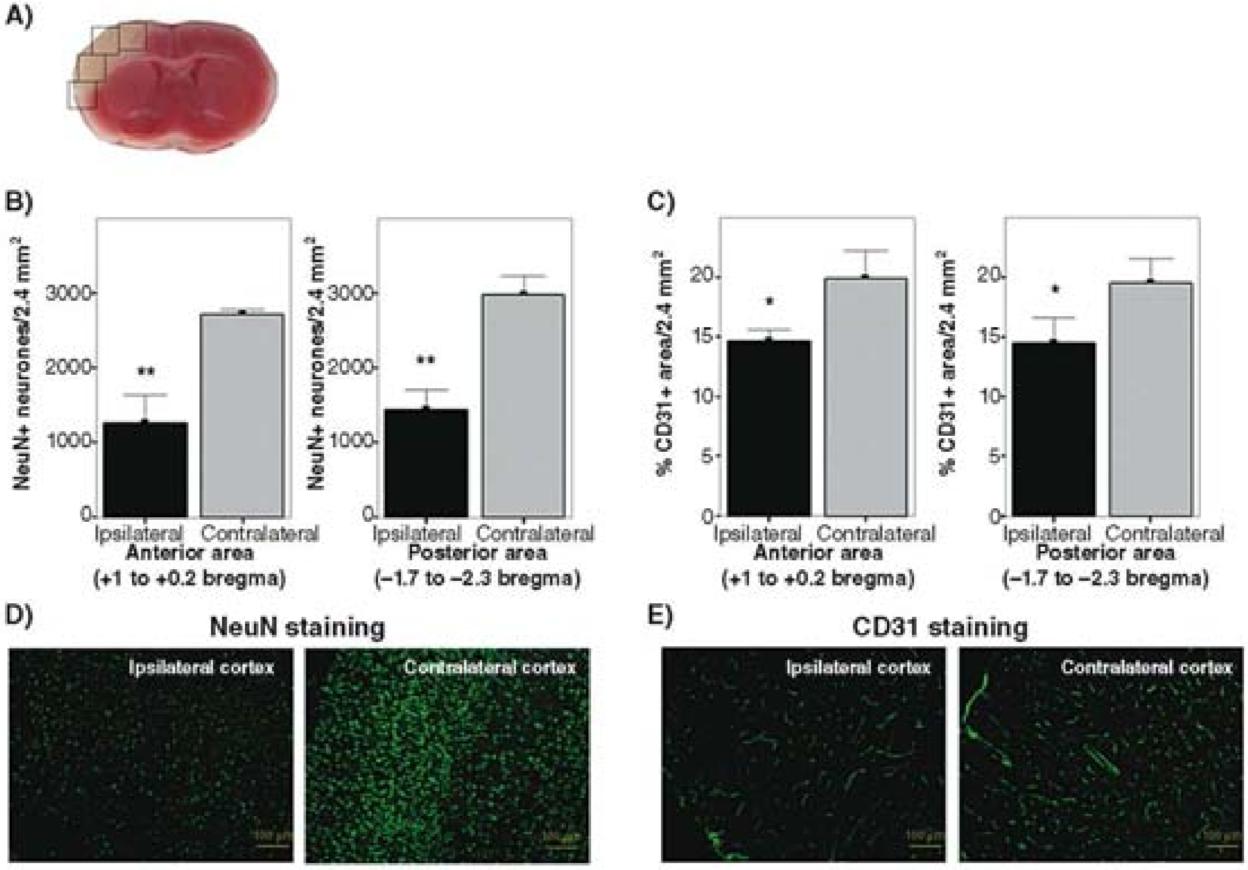

Figure 3. Immunostaining of neurones and vessels. (**A**) NeuN-positive cells and CD31-stained area were calculated in the ipsilateral and corresponding contralateral cortex of animals submitted to transient distal middle cerebral artery occlusion (tdMCAO). Twenty-four hours after 60-min transient ischaemia, a reduced number of neurones (**B**) and decreased CD31-positive area (**C**) were detected within the infarcted ipsilateral as compared to the contralateral area (*$P < 0.05$ and **$P < 0.01$); bar graphs represent mean ± SD. Representative images show ipsilateral decreased NeuN (**D**) and CD31 (**E**) staining compared to the healthy contralateral cortex.

showed an enlarged cell body and a strongly Iba-1-positive staining was detected in the ipsilateral cerebral cortex where the ischaemic insult was applied. This activation was not seen in the corresponding contralateral hemisphere.

After 60 min of transient dMCAO and 24 h of reperfusion, NeuN-stained mature neurones within the infarcted area were significantly reduced as compared to the contralateral hemisphere both in anterior (1273 ± 369 *vs.* 2707 ± 74, $P = 0.003$) and posterior (1445 ± 270 *vs.* 2980 ± 267, $P = 0.002$) areas of the brain, as seen in Figure 3**B** and **D**. Regarding endothelial cells, a reduction in CD31-stained area was also detected within the infarcted cortex as compared to the same region of the contralateral hemisphere, in both anterior (14.68 ± 0.9% *vs.* 19.91 ± 2.3%, $P = 0.022$) and posterior (14.52 ± 2.1% *vs.* 19.51 ± 2.0%, $P = 0.041$) areas, as seen in Figure 3**C** and **E**. Regarding glial cells, reactive astrocytes with increased GFAP expression appeared in the ischaemic ipsilateral cortex, as seen in Figure 4**A**. This GFAP staining showed some astrocyte bodies and multiple cytoplasmatic prolongations, and was not detected in the contralateral hemisphere cerebral cortex (Figure 4**A**). Finally, Iba-1 microglial cells showed activation and mobilization in the infarct boundary as compared to the contralateral hemisphere and infarcted areas (Figure 4**B**).

Functional outcome

Mice subjected to 60-min transient dMCAO displayed a significant sensorimotor behavioural deficit on the corner test at 24 and 48 h after ischaemia, when

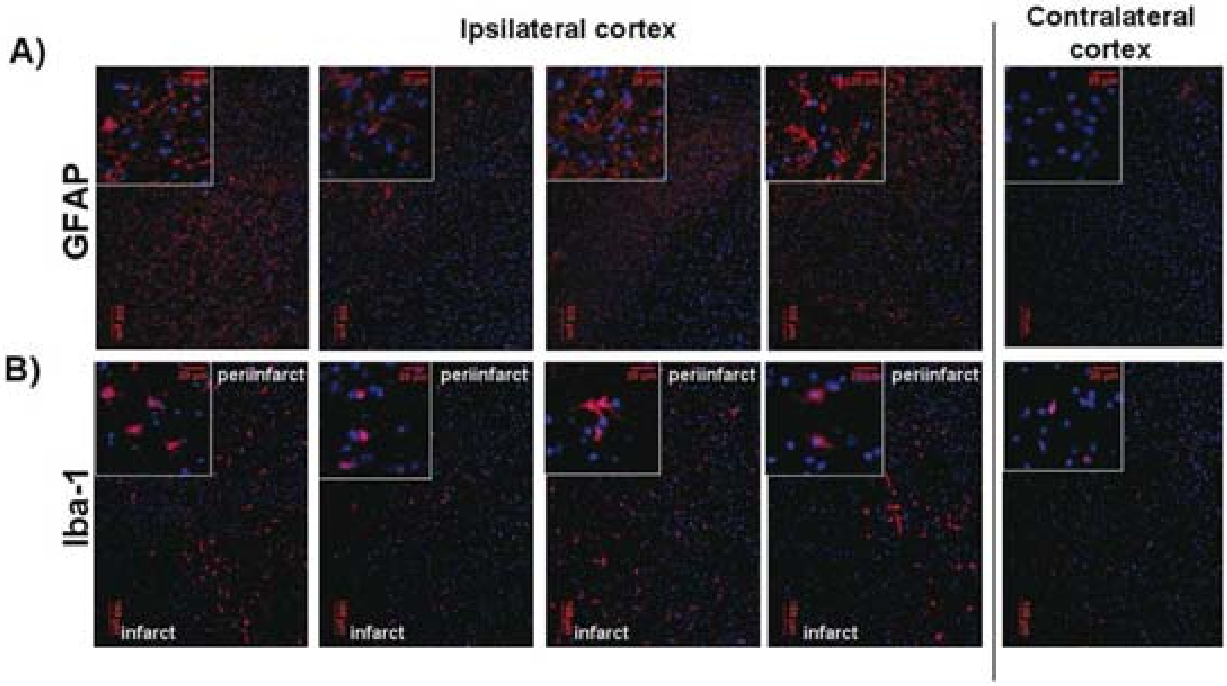

Figure 4. Immunostaining of glial cells. GFAP staining (red) showed reactive astrocytes in the infarcted area of the animals subjected to 60-min transient distal middle cerebral artery occlusion (dMCAO) (A). In the same group activated microglial cells (red) were detected in the infarct boundary (B). Insets show high magnification details of the micrographs. Representative images show no changes in the healthy contralateral cortex. Cell nuclei are stained in blue.

compared to pre-ischaemia scores. In those animals, the laterality index was affected at 24 h compared to pre-ischaemia (−0.68 ± 0.05 *vs.* 0.1 ± 0.2, $P = 0.008$) and at 48 h after transient dMCAO (−0.55 ± 0.31 *vs.* 0.1 ± 0.2, $P = 0.031$), showing a clear asymmetry in turning preference, as seen in Figure 5. At 7 and 14 days after ischaemia, we could not detect neurological impairment as the symmetry was restored (0 ± 0.34 *vs.* 0.1 ± 0.2, $P = 0.994$, and 0.35 ± 0.21 *vs.* 0.1 ± 0.2, $P = 0.772$ respectively).

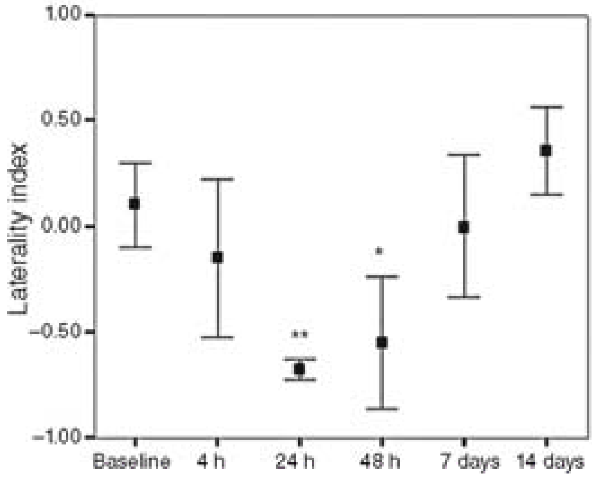

Figure 5. Functional outcome by corner test performance. Mice subjected to 60 min of transient distal middle cerebral artery occlusion (dMCAO) showed an asymmetry in turning preference, demonstrating an impaired functional outcome at 24 and 48 h after reperfusion ($n = 4$; $^*P < 0.05$, $^{**}P < 0.01$ *vs.* baseline). Values represent mean ± SD.

Discussion

In this study, we present a new method for focal transient ischaemia induction in mouse, performed with a single vessel occlusion, low mortality, highly reproducible infarct volumes and well-controlled arterial reperfusion.

There are several experimental models of ischaemic stroke and some of the most frequently used are based on the mechanical occlusion of the proximal MCA [22]. In the distal occlusion models, as the one used here, the damage is restricted only to the cerebral cortex and is highly reproducible. Previous studies have permanently occluded the MCA by electrocoagulation or transiently using a clip or a suture in different mice and rat strains [23,24]. Occluding the artery in transient models with a clip or suture ligation is likely to produce mechanical damage to the vessel, a higher risk of haemorrhage, brain injury or compromised reperfusion. In mice, these complications might be more common due to the reduced size of the artery at its distal segment. In a recent publication by Arsava and colleagues [25], a new model of transient focal ischaemia in mice has been reported by arterial compression to achieve the induction of selective neuronal necrosis. In this work, the authors performed the compression of the MCA for 15 min, which led to localized neuronal damage in the cortex [25]. The authors presented the method as an animal model for transient ischaemic attacks, whereas for compressions of 20 min or longer, they only reported widespread glial as well as neuronal damage, but they did not define it as a model of focal cerebral ischaemia. Based on the arterial compression method, our model produces a consistent ischaemic insult, causing cell damage and impaired functional outcome, and reproduces the result of a complete reperfusion. The present model has the advantages of minimal damage to the vessel and underlying tissue, technical easiness, high reproducibility and low mortality.

The use of laser Doppler flowmetry guidance is necessary for the reliability of the model to ensure a proper 60-min occlusion and posterior reperfusion with no visible damage of the vessel or the surrounding tissue. In this sense, our method follows the guidelines of the STAIR Group regarding laser Doppler flowmetry monitoring [26]. The method showed a high reproducibility for CBF reduction and reperfusion as it was easily accomplished in 94% of the animals.

The TTC staining of brain tissue is the most widely used technique to assess brain injury after cerebral ischaemia in experimental models. In our study, we observed no infarct lesion by TTC staining after 15-min ischaemia, and this led us to look for longer dMCAO occlusion periods to generate an established infarct. The duration of ischaemia has an important influence on the final infarct volume or oedema as has been shown in several rodent models [27,28]. However, the extent of the injury in terms of infarct volume was similar between 60-min transient dMCAO and permanent dMCAO model after 24 h, pointing out that the period between 15 and 60 min is probably critical for cell survival in this model. Cell death mechanisms after ischaemia involve the interaction of apoptotic and necrotic pathways, and are not equally activated in transient and permanent ischaemia [29]. In 'transient' ischaemia, the final injury is the result of the ischaemic and the reperfusion damages and it is known that reoxygenation during reperfusion provides a substrate for numerous enzymatic oxidation reactions and exposes the cells to potential toxins as reactive oxygen species and proinflammatory mediators [30,31]. Therefore, further molecular and biochemical studies are needed to examine the activation of those pathways in our model, and we might hypothesize that some will be different from those pathways activated in the permanent model. In spite of this limitation, the data presented for the permanent ischaemia provide us with information about the final extent of the lesion in our transient model compared to a stable and well established permanent model. Future studies of neuroprotection involving the present cerebral ischaemia model should determine the specific mechanisms involved in brain injury and cell death, which must be investigated to deeply understand the precise mechanisms related to ischaemia reperfusion and potential neuroprotection actions. In addition to the duration of ischaemia, mouse strain also influences the infarct volume as described in previous studies [32]. Balb/c mice have been shown to have larger infarcts than other strains in distal MCAO [33,34]. As differences between strains in the permanent model have been described, it is expected that the transient method described in the present study might have to be adjusted in terms of duration of the ischaemia if different strains are used.

Tissue injury was also assessed using immunohistochemical staining of neurons and vessels at 24 h of reperfusion. A previous study that examined the chronology of infarct lesions in an intraluminal transient MCAO model in mice demonstrated that the peak of damage occurred 24 h following reperfusion [35]. Although our model produces a cortically located infarct, the activation of the pathophysiological mechanisms of cell damage follows the same timing leading to an early peak of damage [36]. Neuronal injury in the ipsilateral infarcted tissue was confirmed by NeuN staining as a large decrease of the number of neurons was observed after transient ischaemia. This result is consistent with previous reports where the peaks of apoptosis and neuronal cell death are detected at 24–48 h after the ischaemic insult, in both rats and mice [35,37]. Although less severe, we also observed a loss of vessels in the same area. Endothelial cells are known to be more resistant than neurones to ischaemic insults, as several in vivo and in vitro studies have shown [38,39]. However, our study demonstrates that the vascular network is highly compromised within the infarct areas indicating the importance of targeting endothelial cells and vessel formation in potential neuroreparative treatments for stroke. Astrocytes and microglial cells also suffered changes after the ischaemic insult. An increase of the GFAP expression characteristic of reactive astrocytes with larger cell bodies was seen as described by others [40]. As previously described, astrocytes are more resistant to ischaemia than neurones, and after a brain injury the surviving astrocytes of the affected region begin to exhibit hypertrophy and proliferation [41,42]. With regard to microglial cells, it has been described that they firstly respond to cerebral ischaemia and transform to activated microglia, with enlarged and amoeboid cell bodies, and appear in the periinfarct areas as early as 30 min after MCAO [43,44]. In accordance, we have seen an activation of the microglia in the ipsilateral cerebral cortex of animals subjected to 45-min transient dMCAO even before the infarct was established. As microglial cells are activated after an inflammatory process, we cannot rule out the possibility that ipsilateral craniectomy and brain surgery might be influencing microglial activation. After 60-min transient dMCAO, microglial activation at the infarct boundary persists although decreased microglial cells have been detected in the infarct areas probably as a consequence of massive cell death in the acute phase of stroke [45].

An important factor when conducting a focal ischaemia model is the monitoring and control of physiological variables [32]. Changes on animal physiological parameters during the surgical procedure may have an influence on lesion extension. In this study, we measured the physiological parameters before ischaemia and during reperfusion by finding no changes due to the surgical procedure. Mortality is another critical parameter in experimental studies as low mortality rates are important to reduce economic costs, the number of animals and to avoid selective bias of the obtained results. Moreover, we were able to assay functional outcome after transient ischaemia, showing a neurological impairment within the first 48 h of reperfusion, similar to those reported in other models of transient MCAO [46]. The corner test showed sensitivity in our 60-min transient dMCAO model within the first 48 h as mice displayed asymmetry in turning preference. However, at days 7 and 14 after transient dMCAO, the test was not able to detect any affectation on the functional outcome. Other studies have reported similar results in a permanent model of dMCAO where C57BL mice showed asymmetry 1 day after ischaemia but not from day 8 [47]. Although our results seem to indicate that an endogenous recovery occurs, further studies are needed to conclude if the animals completely recover from the ischaemic injury by compensation mechanisms or if the corner test is not sensitive enough, as proposed by other authors [48]. Other studies in distal models, both transient and permanent, using different mice strains have reported differences in the turning preference or even no effect in the corner test [47–49]. These results are probably related to anatomical and functional brain differences among strains used and to differences in the extension of the infarcted tissue.

In summary, we have set up a highly reproducible and low-invasive model of focal transient cerebral ischaemia in mice by distal compression of the MCA. After 60 min of ischaemia and 24 h of reperfusion, animals show consistent and reproducible cortical infarcts, impaired short-term functional outcome and severe tissue damage.

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4. DISCUSSIÓ

4.1 El paper dual de les MMPs en l'ictus

Diferents articles han mostrat una relació entre els nivells d'algunes MMPS i la isquèmia cerebral, a nivell de la lesió cerebral, la permeabilitat de la BHE, les transformacions hemorràgiques o la mort cel·lular, però també amb la plasticitat de la matriu extracel·lular i la remodelació de teixits danyats (139,163).

Molts grups han demostrat en models experimentals d'isquèmia cerebral en rosegadors, que els nivells diverses MMPs augmenten ràpidament al cervell isquèmic. Per exemple, després de la isquèmia cerebral es va descriure una expressió anormal de MMP-2 o MMP-9, el que contribueix a la lesió cerebral i la ruptura de la BHE (140,164,165). Altres MMPs, com la MMP-3 i la MMP-13 també s'han trobat sobreexpressades en el teixit isquèmic (144,156). A més, tant l'hemorràgia associada al rt-PA com l'edema cerebral es relacionen amb la desregulació dels nivells de MMP9, complicacions que disminueixen en inhibir aquesta MMP amb l'inhibidor BB-94 (166,167). El rol de les MMPs en la degradació de la làmina basal i el conseqüent debilitament de la BHE explica la seva relació amb la transformació hemorràgica. El nostre grup va identificar una infiltració de neutròfils carregats de MMP-9 en microvasos cerebrals associat a una disminució del col·lagen IV de la làmina basal i a la infiltració d'eritròcits, com també l'alliberació de MMP-9 i la degranulació en neutròfils tractats amb rt-PA (147,168)

Degut a la seva activitat patogènica, les MMPs han estat una diana terapèutica per a la neuroprotecció en l'ictus isquèmic com revisem en l'article 1 d'aquesta tesi doctoral. S'han sintetitzat diferents inhibidors de les MMPs que han demostrat en models animals reduir la mida de l'infart, l'apoptosi i el dany de la BHE. Alguns d'aquests inhibidors, com el BB-94, el BB-1101 o el GM6001, s'anomenen d'ampli espectre perquè bloquegen l'expressió o activació de totes les MMPs (148,166,169). També s'han estudiat inhibidors específics de les gelatinases (MMP-2 i 9), com el SB-3CT, o inhibidors de molècules implicades en les vies de regulació de les MMPs com la ciclooxigenasa (COX), la NADPH oxidasa o l'enzim convertidor d'angiotensina (ACE) (170-173). Els diferents resultats van mostrar que la inhibició de les MMPs després de l'ictus podria esdevenir una teràpia beneficiosa. Tanmateix caldria tenir en compte els efectes a llarg termini d'aquesta inhibició. A dia d'avui no s'ha realitzat cap assaig clínic per a inhibir les MMPs en pacients d'ictus. Tanmateix, s'han realitzat diversos estudis en altres patologies com el càncer per al seu potencial anti-angiogènic. La majoria dels inhibidors de les MMPs que es van testar no van demostrat eficàcia i en alguns casos van mostrar toxicitat i efectes secundaris com dolor muscular (137,174). De tots els inhibidors de les MMPs els únics aprovats per la *Food and Drug Administration* dels Estats Units són antibiòtics com la doxiclina i la minociclina, per exemple per a la prevenció de la periodontitis (175).

Les MMPs participen en el desenvolupament del sistema nerviós central, i algunes d'elles s'hi troben implicades directament (com ara la MMP-2, -3 i -9) ja que s'expressen en el còrtex cerebel·lós en desenvolupament i dirigeixen la migració de les neurones granulars com també participen en la formació d'espines dendrítiques i sinapsis de les cèl·lules de Purkinje (176). De manera paral·lela, les MMPs també regulen el desenvolupament del sistema nerviós de forma indirecta, per exemple, regulant la conversió de les proformes de BDNF i del factor de creixement nerviós (NGF) a les seves formes madures (177). En el cervell adult sa les MMPs estan implicades en processos de remodelació de la matriu extracel·lular, en la regulació de la plasticitat sinàptica i dels processos d'aprenentatge i memòria (178).

Per tant, esperaríem que les MMPs participin també en els processos de reparació cerebral i que la seva inhibició a llarg termini pogués afectar la recuperació després de l'ictus. Alguns estudis realitzats en aquest sentit han mostrat que la inhibició persistent de les MMPs en models animals d'isquèmia cerebral disminuïa la recuperació funcional i augmentava el dany cerebral (179,180). En relació a aquestes observacions s'ha demostrat la participació de les MMPs en la neurogènesi després de l'ictus. Les MMP-2 i -9 han demostrat ésser necessàries per a la correcta migració dels progenitors neurals *in vitro* on la secreció per part de les cèl·lules endotelials d'aquestes metal·loproteïnases té un efecte promotor sobre aquesta migració (154). A més, la MMP-9 també és important en la divisió cel·lular. Sans-Fons i col·laboradors van demostrar que la inhibició de la MMP-9 impedia l'entrada en mitosi de cèl·lules de neuroblastoma (una línia cel·lular amb capacitat de diferenciar-se a cèl·lula neuronal) així com la seva proliferació (181). Altres estudis *in vivo* han mostrat la participació de la MMP-3 i -9 en la migració induïda per quimiocines de les cèl·lules neurals progenitores, així com el paper clau de les MMPs en la migració dels neuroblasts des de la zona subventricular, migració que es redueix si inhibim les MMPs (153,155). Així mateix, l'expressió de MMP-9 s'ha trobat incrementada en el còrtex del peri-infart dies després de la isquèmia (entre 7 i 14) associada a marcadors tant de plasticitat sinàptica com de remodelat vascular, suggerint també una relació entre l'expressió de MMPs i els processos d'angiogènesi (180). En aquest mateix estudi es va comprovar que la inhibició de les MMPs disminuïa l'expressió de VEGF en el peri-infart, confirmant el rol de la MMP-9 en l'alliberament i activació d'aquest factor pro-angiogènic (150).

La implicació de les MMPs, i en especial la MMP-9, en l'angiogènesi ha estat també estudiada en d'altres processos patològics. En models animals d'isquèmia perifèrica, la deficiència de MMP-9 (en ratolins genèticament modificats) ha demostrat disminuir significativament l'angiogènesi, així com la mobilització de les EPCs (160,161). També en estudis del creixement tumoral s'ha trobat una elevada expressió de MMP-9 a les cèl·lules tumorals, així com una disminució de l'angiogènesi tumoral en animals deficients en MMP-9 (182,183). Tornant a la patologia cerebral isquèmica, Hao i col·laboradors van mostrat en un model experimental que la MMP-9 expressada per les cèl·lules mare derivades de medul·la òssia era clau pel procés d'angiogènesi estimulant la mobilització i guiatge de les cèl·lules induïda per VEGF (184).

En aquest sentit aquesta tesi intenta avaluar el potencial terapèutic de les EPCs i dels seus factors per vies paracrines, com també determinar el paper de la MMP-9 en la seva funció en del context de la isquèmia cerebral.

4.2 Els factors secretats per les EPCs posseeixen un potencial angiogènic similar al de les pròpies cèl·lules en el tractament de la isquèmia cerebral

Des del moment de la seva identificació per Asahara i col·laboradors, les EPCs varen ser unes clares candidates des de el primer moment per al tractament de malalties isquèmiques, com van demostrar els propis autors en un model d'isquèmia perifèrica en ratolí (51,185). En models animals d'isquèmia cerebral, diversos autors han demostrat que l'administració tan d'*early* EPCs com d'OECs desprès de l'esdeveniment isquèmic redueix el volum de l'infart i el dèficit funcional a mig termini (a partir del 7è dia) (82,83). En un d'aquests estudis, després de l'administració per via endovenosa de $4x10^6$ OECs marcades radioactivament en rates isquèmiques, es va estudiar la seva distribució. La majoria de les cèl·lules es van trobar al fetge, als ronyons i a la melsa, mentre que molt poques cèl·lules es van arribar al cervell. A més, es van detectar molt poques OECs de les que s'havien administrat incorporades en els vasos de nova creació (83). Malgrat que molt poques EPCs van arribar al cervell i es van incorporar als vasos, l'efecte del tractament va ser contundent, produint un augment de l'angiogènesi i la neurogènesi i una disminució de l'apoptosi al peri-infart (83). De la revisió bibliogràfica d'altres estudis en que s'administren EPCs per via endovenosa també se'n deriva que el nombre de cèl·lules que s'implanten al cervell és extremadament baix en relació al número que s'administren (186,187). Aquest fet també es repeteix en altres tipus cel·lulars, com els precursors neurals. Un estudi per ressonància magnètica en un model d'isquèmia cerebral en rates va mostrar que, tot i produir-se una recuperació funcional en els animals administrats amb cèl·lules, les cèl·lules trasplantades no s'implantaven en els circuits neuronals (188). En conclusió, de l'elevat nombre de cèl·lules que s'administren per via sistèmica, molt poques d'elles arriben al cervell. Harting i col·laboradors van demostrar que només un 0.0005% de les cèl·lules mare mesenquimals administrades per via endovenosa s'implantaven al parènquima cerebral en un model murí de traumatisme cerebral (189).

Per tant els resultats suggereixen que l'efecte de la teràpia cel·lular amb EPCs pugui deure's més a un efecte paracrí de les cèl·lules administrades que a la pròpia incorporació de les cèl·lules en els nous vasos. La importància de l'efecte paracrí en els beneficis de la teràpia cel·lular en l'ictus també s'ha descrit en altres tipus cel·lulars com les cèl·lules mare mesenquimals o les neurals (31,77). Andres i col·laboradors han demostrat recentment com els factors VEGF i TSP-1 i -2 secretats per les cèl·lules mare neurals potencien la plasticitat sinàptica (77). Tenint en compte aquests resultats, una de les qüestions que vam voler adreçar en aquesta tesi era si el tractament amb els factors de secreció de les EPCs, que conté multitud de factors de creixement, inhibidors de l'angiogènesi, quimiocines o citocines, podria tenir el mateix efecte en la recuperació després de la isquèmia cerebral que la teràpia cel·lular.

Prèviament s'havia demostrat el potencial terapèutic dels factors secretats per les EPCs in vitro en un model d'isquèmia perifèrica en rates per a potenciar la neovascularització (107). També en un model porcí d'infart de miocardi, l'administració tan d'EPCs com del seu medi condicionat (medi que ha estat en contacte amb les cèl·lules *in vitro* i que conté els factors que han secretat) van demostrar millorar la funció sistòlica i augmentar la mida dels cardiomiòcits de forma similar (190). A partir d'aquests estudis sabem que el secretoma d'aquestes poblacions d'EPCs conté, entre d'altres factors, factor de creixement de la insulina 1 (IGF-1), factor de creixement transformant beta 1 (TGFβ1), IL-8, SDF-1, HGF, angiogenina, factor de creixement derivat de plaquetes BB (PDGF-BB) o VEGF (107,190). Un estudi previ del nostre grup també va mostrar que les EPCs provinents de sang perifèrica de pacients d'ictus secretaven diferents factors de creixement, com per exemple HGF i VEGF. En el treball presentat en aquesta tesi (article 2) vam estudiar 4 factors pro-angiogènics, identificant la presència en el medi condicionat administrat als ratolins del factor de creixement de fibroblasts bàsic (FGF-b), PDGF-bb i VEGF, mentre que no es va detectar la presència de HGF.

En el nostre estudi, els tractaments (EPCs o el seu medi condicionat) es van administrar per via endovenosa aproximadament 30 hores després de la isquèmia cerebral, una finestra clínicament rellevant. El temps de tractament passada la fase hiperaguda es va triar per poder demostrar la utilitat en la translació a la pràctica clínica un temps de tractament que permetria poder aïllar les pròpies cèl·lules del pacient i efectuant doncs un trasplantament autòleg, com també per tenir una àmplia finestra terapèutica. El nostre estudi és el primer en avaluar l'administració tardana d'EPCs en comparació amb la dels seus factors en la isquèmia cerebral, i demostra un increment de l'angiogènesi en les zones del peri-infart corticals en ambdós tractaments *versus* animals no tractats o no isquèmics. Com en els estudis previs, el medi condicionat de les EPCs va demostrar ser tan efectiu com les cèl·lules soles i fins i tot produir un major increment de l'angiogènesi (tot i que aquesta diferència no va ser significativa). Els factors que hem identificat en el medi condicionat ja havien demostrat en d'altres treballs potenciar la neovascularització després de la isquèmia. Per exemple, l'administració tardana de VEGF va demostrar augmentar l'angiogènesi en el cervell isquèmic i en models d'isquèmia perifèrica l'administració conjunta de FGF i PDGF-bb va incrementar la vascularització i el flux sanguini a l'àrea afectada (57,191,191). Els resultats mostrats en l'administració tardana obre la porta a nous estudis sobre la possibilitat de poder aconseguir un major benefici terapèutic administrant els tractament més aviat després de la isquèmia. En aquest cas seria especialment interessant disposar d'un tractament basat factors.

Avui en dia és reconegut l'estret lligam en les àrees del peri-infart entre els processos d'angiogènesi i neurogènesi (64). La secreció per part de les cèl·lules vasculars de factors de creixement a l'anomenat nínxol neurovascular estimula la migració dels neuroblasts, migració que es produeix associada als vasos (64). També sabem que l'administració de diferents tipus cel·lulars ha demostrat en models animals d'isquèmia cerebral potenciar ambdós processos. El trasplantament intracerebral de cèl·lules mare mesenquimals derivades de medul·la òssia va resultar en un increment de l'angiogènesi i de la proliferació, migració i diferenciació de progenitors neurals (192). L'administració endovenosa de cèl·lules mare mesenquimals o EPCs també ha demostrat potenciar tant l'angiogènesi com la neurogènesi en el teixit cerebral afectat (83,193). En els resultats que presentem en aquesta tesi (article 2) l'efecte dels tractaments sobre la neurogènesi no va ser tan evident com en el cas de la neovascularització. Els animals tractats amb medi condicionat van mostrar un lleuger increment en el número de neuroblasts en l'àrea dorsolateral de la zona subventricular, suggerint un efecte potenciador en la migració dels precursors neurals també identificat per d'altres autors (47). En canvi, l'administració d'EPCs va incrementar moderadament la reorganització axonal en el peri-infart. Aquest remodelat de la substància blanca s'associa amb la recuperació de la funció cerebral i per tant una millora funcional (194,195). Aquests resultats també es van acompanyar d'una millora funcional estadísticament significativa en ambdós grups de tractament que no es va produir en el grup control: tant els animals tractats amb EPCs com amb medi condicionat van mostrar una recuperació en la força i habilitat de les extremitats anteriors que s'havia vist significativament reduïda per la isquèmia cerebral. Tot i això els animals isquèmics no tractats van mostrar una lleugera recuperació espontània.

Els resultats d'aquesta tesi demostren doncs el potencial neuroreparador de les EPCs però també dels factors de secreció de forma independent. Davant l'evidència que molt poques de les cèl·lules que s'administren arriben al cervell i s'implanten en el teixit, els nostres resultats recolzen la hipòtesi que els efectes neuroreparadors de la teràpia cel·lular podrien atribuir-se parcialment a l'estimulació que es produeix en el teixit afectat a través de la secreció de factors tròfics potenciant els processos endògens d'angiogènesi i neurogènesi (66).

Substituir el trasplantament cel·lular per estratègies lliures de cèl·lules però basades en el seu potencial terapèutic ens permetria millorar alguns aspectes negatius que es poden derivar de l'administració de cèl·lules vives. Qualsevol trasplantament de teixits o cèl·lules pot produir complicacions derivades d'una reacció immunitària (196). Tanmateix, en relació al possible rebuig del trasplantament, les EPCs, a diferència de les cèl·lules endotelials madures, han demostrat tenir una baixa immunogenicitat també quan es diferencien en cèl·lules endotelials, fent-les per tant unes excel·lents candidates per a la teràpia cel·lular (197). Una altra font de complicacions són les derivades de l'administració de les cèl·lules. Respecte les possibles vies d'administració, l'administració intracerebral directa sobre les àrees cerebrals diana garanteix l'arribada de les cèl·lules però suposa una aproximació més agressiva pels pacients d'ictus amb uns certs riscos vitals i uns elevats costos econòmics degut a les condicions en s'haurien de realitzar aquestes administracions. Tot i els possibles efectes de l'administració per via endovenosa o intraarterial, on es produeix un filtratge per diferents òrgans (pulmons, fetge), es poden generar èmbols i obstruir la vasculatura o on les cèl·lules poden arribar a d'altres òrgans on en certes situacions patològiques podrien proliferar produint tumors, aquestes serien les vies d'elecció en front la intracerebral en el cas d'una translació a la clínica (198-200). Per aquests motius la possibilitat de basar el tractament pro-angiogènic amb els factors de secreció de les cèl·lules és de gran interès terapèutic.

En resum, els resultats presentats en aquesta tesi demostren que els factors que secreten les EPCs són tant efectius com a tractament neuroreparador pro-angiogènic amb EPCs en la isquèmia cerebral, on poden aportar avantatges respecte l'administració de cèl·lules des d'un punt de vista translacional. L'aprofundiment en l'estudi del secretoma de les EPCs com dels efectes dels diferents factors identificats sobre els processos de neuroreparació ens ha de permetre definir els temps i dosis òptims d'administració, i proporcionar noves propostes terapèutiques per la fase subaguda o crònica de la malaltia. D'altra banda, no podem deixar de banda l'efecte terapèutic de les cèl·lules que s'implanten al teixit i els beneficis que comportaria una millor distribució, major supervivència, la migració i l'empelt de les cèl·lules trasplantades. Els nostres resultats mostren també una major reorganització axonal en el peri-infart en aquest grup de tractament tot i que probablement estudis a més llarg termini (mesos) permetran valorar millor aquestes observacions. Els nostres resultats deixen també la porta oberta a nous estudis on valorar els efectes de la teràpia combinada entre EPCs i els seus factors de creixement en diferents dosis.

4.3 La isquèmia cerebral estimula l'alliberament de les EPCs així com la seva funcionalitat *in vitro*

Diversos estudis han demostrat que la isquèmia promou la mobilització de les EPCs. L'estímul isquèmic activa l'expressió de factors pro-angiogènics com el VEGF i el SDF-1, que al seu temps estimulen la mobilització de les cèl·lules progenitores des de la medul·la òssia cap a la circulació (201). En un model experimental en ratolins i conills d'isquèmia perifèrica es va observar un augment després de la isquèmia en el número d'EPCs circulants amb un pic màxim al setè dia (202). També, en estudis en humans amb patologies isquèmiques com el traumatisme vascular o l'infart agut de miocardi han mostrat nivells elevats d'EPCs en la sang perifèrica dels pacients (201,203).

En el cas de l'ictus, la mobilització de les EPCs en resposta a la isquèmia cerebral no és una qüestió clara. Alguns estudis han demostrat un increment de les EPCs en pacients respecte als controls (126,132), mentre que en d'altres, els pacients mostraven una disminució dels nivells d'EPCs (124,127). Els resultats publicats pel nostre laboratori on es va realitzar citometria de flux, els resultats van mostrar un augment del nivells d'EPCs en sang perifèrica en pacients d'ictus en la fase més aguda de la malaltia (primeres 24 hores) respecte als controls sans (130). Les diferències en les tècniques utilitzades per l'aïllament i al quantificació dels nivells d'EPCs així com les característiques dels pacients i controls utilitzats en els diferents estudis podrien explicar aquestes divergències. Per tal de poder resoldre aquesta qüestió en l'article 3 presentat en aquesta tesi vam avaluar per primera vegada la mobilització de les EPCs després de la isquèmia cerebral en un model experimental en ratolí on la única variable canviant era la presència o no d'isquèmia cerebral permanent.

La tècnica d'elecció va ser la quantificació a partir de cultius cel·lulars d'*early* EPCs obtingudes de la melsa (que actua com a reservori d'EPCs), ja que el reduït volum de sang dels ratolins limitava l'ús de la citometria de flux (204). Els resultats van mostrar un increment de les EPCs poc després de la isquèmia, l'increment era detectable tan sols 6 hores després de l'esdeveniment isquèmic. Així doncs el nostre model d'isquèmia focal produeix la mobilització de les EPCs al poc temps de l'inici de la isquèmia. Aquest augment en l'alliberació d'EPCs sembla ser transitori ja que a les 24 hores es recuperen els nivells previs a la isquèmia. Aquest perfil temporal és similar a l'observat en pacients amb trauma vascular, que van mostrar un increment en la mobilització d'EPCs entre les 6 i les 12 hores després del dany isquèmic i un retorn als valors basals a les 48 hores (203). També els resultats previs del nostre grup havien mostrat un augment dels nivells d'EPCs mesurades per citometria en pacients d'ictus agut (entre 3 i 24 hores després de la isquèmia) però no es van veure diferències significatives respecte als controls en moments posteriors de la malaltia (130).

A més de la mobilització en termes de recompte d'EPCs també hem estudiat l'efecte de la isquèmia cerebral en la funcionalitat de les EPCs, comparant per primera vegada la capacitat vasculogènica d'EPCs d'animals amb isquèmia cerebral i controls. Els estudis de tubulogènesi *in vitro* presentats en l'article 3 van mostrar un augment significatiu de la funcionalitat en les EPCs provinents d'animals isquèmics, que realitzaven un major nombre d'estructures tubulars que les EPCs provinents d'animals control, dibuixant així una xarxa vascular més extensa. Els nostres resultats d'angiogènesi a temps real (captant una imatge cada 30 minuts) van permetre identificar per primer cop un patró de formació de xarxes vasculars en Matrigel™ en dues fases: la de construcció (en les primeres 10-12 hores) en que les EPCs assoleixen la seva capacitat màxima de tubulogènesi, i una fase de remodelació en la que sense incrementar aquesta extensió màxima al llarg dels temps les EPCs segueixen modificant constantment les connexions entre cèl·lules en un moviment dinàmic constant. Recolzant aquestes dades, un estudi recent en isquèmia cerebral en rates va demostrar que el trasplantament de cèl·lules mare mesenquimals derivades de medul·la òssia sotmeses a isquèmia (hipòxia *in vitro*) millorava la recuperació funcional dels animals respecte al transplantament de les cèl·lules control (205). Un estudi previ també en rates i en el que administraven també cèl·lules mesenquimals, havia mostrat també un increment en la millora funcional i un major augment de l'angiogènesi en els animals que havien rebut cèl·lules provinents de rates isquèmiques (206). En ambdós estudis l'efecte terapèutic de les cèl·lules isquèmiques s'associava amb un augment de la secreció de factors de creixement per part d'aquestes, que explicaria un increment en l'efecte paracrí del tractament. En el nostre estudi les EPCs mostraven ser més funcionals després de la isquèmia, però no podem fer cap associació amb la secreció de factors ja que no vam determinar el seu secretoma. El proper pas seria estudiar si aquesta major funcionalitat de les EPCs isquèmiques es tradueix en una major eficàcia d'una teràpia cel·lular basada en EPCs en un model animal *in vivo*, així com demostrar si l'activació funcional de la isquèmia també es produeix en cèl·lules humanes. En relació a la modulació de la funcionalitat de les EPCs en les diferents fases de l'ictus, un estudi previ del nostre grup ja vam mostrar diferències significatives en la capacitat tubulogènica de les EPCs obtingudes en diferents fases de la malaltia (< 24 hores *versus* > de 24 hores), sent superior en fases més tardanes (130).

Els resultats obtinguts fins al moment ens demostren que la isquèmia cerebral estimula la funcionalitat de les EPCs *in vitro*, així com la seva mobilització *in vivo*, i suggereixen uns possibles millors resultats en trasplantaments de cèl·lules obtingudes de pacients isquèmics ja que podrien ser més funcionals que de cèl·lules provinents de controls en futurs assajos clínics. Com s'ha discutit en l'apartat anterior, la secreció de factors de creixement per part de les cèl·lules és clau per explicar el seu potencial terapèutic i per tant esperaríem un major efecte en el tractament en el cas de cèl·lules que secretin majors nivells de factors, com podrien ser les isquèmiques.

4.4 La deficiència de MMP-9 disminueix el número d'EPCs *in vivo* i compromet la seva funcionalitat *in vitro*

Com hem descrit en la introducció, la MMP-9 és una proteasa clau en la mobilització de les cèl·lules progenitores de la medul·la òssia (119). Un estrés patològic, com podria ser la isquèmia cerebral, mobilitza i recluta ràpidament les cèl·lules des de la medul·la òssia a la circulació tal com hem vist en el cas del nostre model experimental d'isquèmia cerebral per oclusió permanent de l'artèria cerebral mitja a nivell distal (article 3). La MMP-9 també té un paper principal en el procés de migració fins a les àrees afectades a través de la regulació de citocines (119). Per aquest motiu, s'ha descrit que els animals deficients en MMP-9 tenen deficiències en la mobilització de les cèl·lules mare i progenitores, entre elles les EPCs. Aquests animals no responen a l'estimulació de la mobilització d'EPCs en resposta a VEGF, que si que es produeix en animals naïve (119,207).

Els nostres resultats presentats en l'article 3, mostren una disminució dels nivells d'EPCs circulants en animals deficients en MMP-9 en ratolins no isquèmics, confirmant com s'havia vist en estudis anteriors una reducció de la mobilització de cèl·lules progenitores en absència d'aquesta metal·loproteïnasa (119). Tanmateix, els nostres resultats mostren que 6 hores desprès de la isquèmia els nivells d'EPCs continuen disminuïts en ratolins deficients per MMP-9 respecte els seus *wild-type*, en canvi a les 24 hores els nivells entre els dos genotips són similars. Per tant, el pic en el nombre d'EPCs que s'observa en animals isquèmics WT a les 6 hores es veu desplaçat a les 24 hores quan hi ha deficiència de MMP-9. Aquests resultats, a banda de vincular la presència de MMP-9 amb la mobilització d'EPCs també suggereixen que en el nostre model d'isquèmia poden existir altres vies de vies de mobilització de les EPCs que no són dependents de MMP-9 ja que finalment si que es produeix la mobilització de les EPCs malgrat la deficiència de MMP-9. Hi ha diferents vies de senyalització implicades en la mobilització de les cèl·lules mare progenitores, algunes mediades per MMPs però també per altres proteïnases com la elastasa o la catepsina G (208). S'ha demostrat per exemple que el G-CSF activa la mobilització de les cèl·lules progenitores des de la medul·la òssia per vies dependents d'elastasa i catepsina (209). No coneixem bé quines vies són les que activa el nostre model d'isquèmia cerebral en els ratolins deficients de MMP-9, i l'anàlisi dels factors angiogènics que es puguin activar de forma diferenciada en aquests animals respecte als animals WT seria d'interès en estudis futurs.

A més del seu paper en la mobilització de les cèl·lules mare i progenitores, la MMP-9 també és necessària per la neovascularització. Estudis previs van demostrar en un model d'isquèmia perifèrica que en animals KO en MMP-9 es produïa una inhibició de l'angiogènesi i una disminució del flux sanguini en l'àrea afectada (160,161). Aquesta deficiència era compensada quan es trasplantava la medul·la òssia d'animals WT als animals deficients en MMP-9 (161). En el nostre estudi (article 3), les EPCs provinents d'animals deficients en MMP-9 han demostrat menor capacitat tubulogènica en els estudis de funcionalitat *in vitro*. L'absència de MMP-9 provoca que tant les cèl·lules control com isquèmiques produeixin un menor nombre d'estructures tubulars que les cèl·lules WT. Tot i que en el mateix estudi demostrem que la isquèmia produeix un increment de la funcionalitat en les EPCs, en aquelles cèl·lules obtingudes d'animals KO en MMP-9, aquest increment és molt moderat i no es tradueix en una diferència significativa com ho era en el cas de les cèl·lules WT. Per tant, els resultats suggereixen que l'augment de la tubulogènesi després de l'estímul isquèmic en les EPCs depèn de la MMP-9. El nostre estudi va més enllà i demostrem aquesta disminució en les habilitats angio-vasculogèniques en inhibir farmacològicament les MMPs i específicament la MMP-9, tant en EPCs d'animals WT com en cèl·lules obtingudes d'humans sans. Estudis previs havien mostrat resultats similars (161). L'efecte de la MMP-9 en la vasculogènesi també s'ha avaluat en cèl·lules mamàries tumorals. La línia cel·lular mamària tumoral resistent a adriamicina expressa MMP-9 constitutivament i aquesta expressió és necessària per a la formació de tubs (210). D'altra banda, la línia cel·lular sensible a adriamicina (SCMF-7), que no expressa MMP-9, no pot realitzar estructures tubulars. L'exposició de les cèl·lules SMCF-7 a MMP-9 recombinant humana indueix la migració cel·lular però no la formació de xarxes tubulars. Aquestes dades es corresponen amb les obtingudes en les EPCs d'animals deficients en MMP-9 tractades amb MMP-9 recombinant o amb el medi condicionat de les cèl·lules WT (que conté MMP-9), tractaments que no van poder revertir la seva incapacitat per a formar estructures vasculars. Els resultats d'aquesta tesi demostren el paper clau de la MMP-9 endògena en el desenvolupament de les capacitats angiogèniques de les EPCs. A partir d'aquestes dades caldrien més estudis per dilucidar els mecanismes exactes implicats en aquests processos vasculogènics dependents de MMP-9.

La importància de la MMP-9 en la vasculogènesi de les EPCs *in vitro* que mostren els resultats d'aquesta tesi sembla que també es podria traduir en un deficiència en la vascularització després de la isquèmia cerebral com s'ha vist anteriorment en models d'isquèmia perifèrica (160,161). Tanmateix, sembla que el paper de la MMP-9 en la vascularització podria ser diferent segons el teixit afectat. Per exemple, en el cas de l'infart de miocardi es va veure una estimulació de l'angiogènesi en els animals KO en MMP-9 (211). L'explicació que suggereixen els autors és que la MMP-9 seria necessària per la vascularització de teixits que no estan altament vascularitzats com el múscul, l'ull o la medul·la espinal mentre que la seva presència seria deletèria en òrgans altament vascularitzats com el cor o els tumors en etapes tardanes. Respecte al cervell, en un model d'isquèmia cerebral en rata es va comprovar que la inhibició farmacològica a llarg termini de les MMPs disminuïa els processos de reparació, i es va demostrar el rol essencial de la MMP-9 en aquests processos (180). Tot i la publicació d'alguns estudis utilitzant inhibidors farmacològics, no coneixem cap treball sobre la recuperació endògena després de la isquèmia cerebral en animals deficients en MMP-9. Caldria desenvolupar aquest tipus d'estudis per a confirmar el paper d'aquesta metal·loproteïnasa en l'angiogènesi i la neurogènesi i la possible aparició de mecanismes compensatoris. Finalment també és necessari aclarir com afectaria a la neuroreparació després de la isquèmia cerebral la deficiència de MMP-9 en les EPCs a partir d'estudis *in vivo* de teràpia cel·lular.

4.5 La importància de la reperfusió i la homologia amb l'ictus humà en els models animals: desenvolupament d'un model d'isquèmia cerebral transitòria

Els models animals d'isquèmia cerebral es van començar a desenvolupar cap al 1970 amb l'objectiu d'identificar els mecanismes responsables del dany tissular i per assentar les bases pe(34)r al desenvolupament de nous tractaments per a l'ictus (34,212). Existeixen diferents models i és important escollir el més adient per a l'objectiu de cada experiment. En els estudis que hem presentat hem treballat amb un model d'oclusió permanent i distal de l'artèria cerebral mitja en ratolí. Aquest model produeix un infart localitzat al còrtex cerebral, és molt reproduïble i amb molt poca variabilitat respecte a la mida i localització de la lesió i té una molt baixa mortalitat, característiques ideals per a la realització d'estudis de neuroreparació (213). S'ha descrit que molts dels processos de neuroreparació com la diferenciació de les cèl·lules progenitores neurals, l'angio-vasculogènesi o la reorganització de les sinapsis i els circuits neurals tenen lloc des del teixit sa del peri-infart (214). El model animal que hem utilitzat també és adequat per l'estudi d'aquests processos ja que ens permet localitzar fàcilment l'àrea afectada per l'infart així com el peri-infart, i el teixit afectat es limita a una sola regió cerebral com és el còrtex (35). Un altre aspecte important en l'elecció del model és que ens permeti avaluar la millora funcional, i per això és necessari que produeixi un dèficit neurològic que puguem mesurar. L'anatomia i fisiologia cortical dels ratolins està ben descrita, i podem conèixer que el nostre model afecta principalment el còrtex sensorial i motor de les extremitats anteriors així com el còrtex somatosensorial que controla la funció de les vibrisses (215). Estudis previs en ratolins així com els nostres resultats de l'article 2 han descrit que la majoria dels tests utilitzats són capaços de mostrar el dèficit neurològic durant les primeres 24 a 48 hores després de la isquèmia però no en temps més tardans en que es produeix una recuperació endògena (216-218). Les explicacions possibles a aquestes dades poden ser que els animals desenvolupen alguns mecanismes de compensació des de l'hemisferi contralateral o que els tests utilitzats no siguin prou sensibles. Per tant aquest és un aspecte avui en dia encara sense resoldre pels models d'isquèmia cortical en ratolí.

El model d'oclusió permanent i distal de l'artèria cerebral mitja que hem utilitzat en els nostres estudis és un model acceptat i utilitzat com demostren els estudis citats anteriorment. Tanmateix, els criteris establerts pel grup STAIR (*Stroke Treatment Academic Industry Roundtable*) consideren que els models d'oclusió permanent són els adients per als primers estudis que caldrà completar amb models d'oclusió transitòria, i per tant amb reperfusió del teixit (26,27). La oclusió permanent és poc freqüent en humans i per tant un model animal amb reperfusió mimetitzaria millor l'evolució de l'ictus en humans. D'altra banda la reperfusió del teixit activa els mecanismes de mort cel·lular necròtica i apoptòtica, de forma diferenciada a una oclusió permanent, ja que en els models d'oclusió transitòria el dany final és el resultat dels processos d'isquèmia i reperfusió (219). A més, els models transitoris, que impliquen el restabliment del flux sanguini després de la isquèmia, serien l'opció a escollir per a estudiar l'efecte sobre el teixit de possibles tractaments (farmacològics o cel·lulars) per tal d'assegurar l'arribada d'aquests a l'àrea afectada. Per tots aquests motius ens era necessari posar a punt un model d'oclusió transitòria i distal de l'artèria cerebral mitja, per a continuar els estudis resultants d'aquesta tesi i que ens permetés tenir un infart cortical ben localitzat i amb poca variabilitat però amb la reperfusió de l'àrea afectada.

Els models que generen infart cerebral per l'oclusió distal i transitòria de l'artèria cerebral mitja establerts fins al moment, utilitzen com a mètodes d'oclusió de l'artèria un clip o una sutura (213,220,221). Es tracta de mètodes més invasius ja que requereixen obrir la duramàter per accedir a l'artèria, cal tenir molta destresa per no danyar l'escorça cerebral tenint en compte la mida tan reduïda de l'artèria en ratolins i sovint danyen l'artèria evitant una correcta reperfusió (32). El mètode que vam desenvolupar oclou l'artèria cerebral mitja per compressió mecànica a través d'una agulla esmussada ("roma"). Aquest mètode permet minimitzar al dany al vas i al teixit cerebral, té una baixa mortalitat durant la cirurgia i a llarg termini i és fàcil i ràpid de dur a terme. Els nostres resultats presentats en l'article 4 mostren que després de 60 minuts d'oclusió de l'artèria per compressió i la posterior reperfusió es produeix un infart ben establert en ratolins de la soca Balb/c, molt similar en mida i localització al del model d'oclusió permanent, amb una reducció significativa del nombre de neurones i vasos i la presència d'astròcits reactius a l'àrea afectada així com una activació de la micròglia en el peri-infart. A més, vam ésser capaços de mesurar dèficit neurològic en els animals isquèmics durant les primeres 48 hores després de la reperfusió, confirmant la presència de lesió cerebral. Com en els estudis realitzats amb el model permanent, el test utilitzat (*corner test*) que avalua l'asimetria en la preferència dels girs que es produeix després de la lesió, no va poder detectar cap afectació funcional a llarg termini. En resum, els resultats presentats demostren que hem posat a punt un nou mètode per a desenvolupar un model d'oclusió distal i transitòria de l'artèria cerebral mitja en ratolí que produeix un infart establert, i que tenint en compte les guies internacionals hauria de ser el proper model experimental per a seguir desenvolupant les nostres aproximacions terapèutiques amb EPCs o els seus factors de secreció en estudis *in vivo*.

5. CONCLUSIONS

Les conclusions d'aquesta tesi són:

- La regulació de les MMPs a curt i llarg termini després de l'ictus és una estratègia terapèutica vigent tant per a la neuroprotecció com per a l'estimulació dels processos de neuroreparació.

- L'administració d'EPCs com dels seus factors de secreció (lliures de cèl·lules) són tractaments efectius que potencien l'angiogènesi i la millora funcional en un model d'isquèmia cerebral.

- La isquèmia cerebral estimula, en un model murí, la mobilització de les EPCs a la circulació perifèrica i resevoris com també la seva funcionalitat *in vitro*.

- La MMP-9 és una proteasa implicada en la mobilització de les EPCs de la medul·la òssia i regula les seves funcions angio-vasculogèniques.

- El mètode d'oclusió distal i transitòria de l'artèria per compressió, caracteritzat per la producció d'una lesió localitzada i reproduïble i la presència de reperfusió, acompleix els criteris necessaris per a estudis de neuroreparació.

6. ANNEX

Article 4

Metalloproteinase and stroke infarct size: role for anti-inflammatory treatment?

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Metalloproteinase and stroke infarct size: role for anti-inflammatory treatment?

Anna Morancho, Anna Rosell, Lidia Garcia-Bonilla, and Joan Montaner

Deregulation of matrix metalloproteinases (MMPs), the largest class of human proteases, has been implicated in brain damage in both animal and human studies. Some MMPs are elevated after stroke (both in plasma and in brain tissue), and their expression is enhanced by t-PA during thrombolysis related to hemorrhagic transformation events. Although the exact cellular source of MMPs remains unknown, brain endothelium, astrocytes, neurons, and inflammatory activated cells, such as neutrophils, may release MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, and/or MMP-13. Neurovascular perturbations occurring after stroke lead to blood–brain barrier leakage, edema, hemorrhage, leukocyte infiltration, and progressive inflammatory reactions to brain injury over hours or even days after the initial stroke. Synthesized MMP inhibitors and several compounds used for stroke secondary prevention, such as anti-inflammatory drugs, might decrease MMPs and improve the acute treatment of human brain ischemia without compromising the beneficial effects of matrix plasticity during stroke recovery.

Keywords: stroke; biomarkers; ischemia; thrombolysis; metalloproteinases

Introduction

Stroke remains a major cause of death and disability worldwide, and its pathophysiology is highly complex. After the cerebral ischemic event, a cellular catastrophe occurs within the hypoxic tissue, leading in a few minutes to severe lesions at the infarction area that may extend through the surrounding tissue owing to secondary cell loss. Nowadays, only thrombolytic therapies with tissue plasminogen activator (t-PA) are given to treat hyperacute ischemic strokes, but only 2–5% of all strokes worldwide are receiving a pharmacological therapy to restore the blood flow. The initial vascular event rapidly leads to energy failure, which ultimately triggers a wide and intricately linked cascade of neuronal death pathways. Over the past decade, several molecular mechanisms involved in neuronal death have been thought to comprise excitotoxicity, oxidative stress, and perhaps even programmed cell death signals such as apoptosis or autophagy.[3] Unfortunately, a decade of monotherapies focused on neuroprotection have not yielded successful treatments for stroke expanding the focus to include other cell types and extracellular matrix components.[1–4] In addition to these primarily intracellular events, an increasing emphasis on the importance of intercellular signaling has emerged in the last decade: all cells in the so-called neurovascular unit are affected, not just neurons.[4–6] Neurovascular perturbations lead to blood–brain barrier (BBB) leakage, edema, hemorrhage, leukocyte infiltration, and progressive inflammatory reactions to brain injury over hours or even days after the initial stroke. Disruption of the BBB in stroke has been classically associated with oxidative stress; oxygen and nitrogen radicals may damage vascular lipids and proteins leading to mechanical leakage in the neurovascular barriers.[6] However, other pathways of neurovascular inflammation may serve to amplify tissue damage after stroke and the deregulation of some proteases with subsequent aberrant proteolysis of neurovascular matrix substrates. Then, once neurovascular barriers are affected, multiple neuroinflammatory cascades become activated, thus potentially leading to further secondary brain injury.[7] These include alterations in cytokine/chemokine profiles, adhesion molecule expression, or protease deregulation.

In this review, we will focus on the pathophysiologic actions of matrix metalloproteinases (MMPs) and the possible benefits of MMP inhibitors treatment for stroke. It is known that an uncontrolled MMP activity may be responsible for the degradation of extracellular matrix (ECM) and basal lamina proteins contributing to initial brain injury after stroke. In contrast, other endogenous MMP responses may turn out to be key mediators in stroke recovery in a later phase of the disease.[8,9]

Matrix metalloproteinases comprise a family of zinc-dependent proteases involved in the regulation of cell–matrix composition; they are known for their ability to cleave one or several basal lamina and extracellular matrix components. More than 25 different secreted- and cell surface-bound MMPs have been described. Apart from being capable of degrading all kinds of extracellular matrix proteins, MMPs can also process a number of bioactive molecules. Although they are products of different genes, these endopeptidades share common structural (such as an amino-terminal propeptide and a catalytic and a hemopexin-like domain) and functional elements.[10] All members of the MMP family are produced in a latent form and become secreted or transmembrane type proteins. The MMPs are initially synthesized and secreted into the extracellular space as inactive zymogens, with a propetide domain that must be cleaved by other proteases, or MMPs before the enzyme is active. Some of the physiological activator proteases are plasmin, t-PA, and urokinase-type plasminogen activator (uPA).[11] The catalytic activity of the MMPs is regulated at multiple levels including transcription, secretion, activation, and inhibition. Namely, inhibition of MMPs is mediated by members of the tissue inhibitor of metalloproteinases (TIMP) family, which currently includes four proteins: TIMP-1, TIMP-2, TIMP-3, and TIMP-4.[10]

Among the multiple functions of the MMPs, they are known to be involved in the cleavage of cell surface receptors, the activation or inactivation of chemokines and cytokines, and the release of apoptotic ligands. They also participate in cell proliferation, migration, differentiation, angiogenesis, and apoptosis.[12] Initially, MMPs were classified by preferred substrate specificities such as collagenases, gelatinases, stromelysins, and matrilysins. However, their substrate specificity is extensive because one single MMP can display distinct molecular interactions with other proteinases and substrates *in vivo* that make their biology unquestionably complex. For example, MMP-3, MMP-7, and MMP-10, members of the stromelysin subclass, can cleave many ECM components, including proteoglycans, fibronectin, collagens, and gelatins. The collagenases, MMP-1, MMP-8, and MMP-13, target primarily fibrillar but also nonfibrillar collagens. The gelatinases, MMP-2 and MMP-9, are also potent in their ability to cleave denatured collagens.[13]

Matrix metalloproteinases and brain damage

Role in neurovascular proteolysis

The standard hypothesis postulates that some MMPs play a central pathologic role in stroke by degrading ECM substrates that are essential for normal signaling and homeostasis within the neurovascular unit. In the context of acute neurovascular injury, MMPs may degrade basal lamina, weaken vessels, and predispose them to leakage and rupture. After human stroke and in experimental models of cerebral ischemia, many MMPs are significantly increased.[14–17] The mechanisms of MMP-mediated brain injury are diverse and occur either directly through degradation of brain matrix substrates or indirectly through activation of other bioactive molecules.

In animal models of cerebral ischemia, MMP expression is increased significantly and related to BBB disruption, edema formation, or hemorrhagic transformation (HT)[14–18] (Fig. 1). Treatment with MMP inhibitors or MMP-neutralizing antibodies reduced edema and infarction in rat and mouse models.[19–20] It was demonstrated that MMP-9 knockout mice had significantly smaller lesions compared to wild-type (WT) mice after permanent and transient focal ischemia, emphasizing the central role of this protease, at least in experimental models.[19] A similar finding was obtained after transient global cerebral ischemia, with hippocampal neuron death being significantly ameliorated in MMP-9 knockout mice.[21] Moreover, MMP-9 was demonstrated to mediate the hypoxia-induced edema formation by a mechanism involving vascular endothelial growth factor (VEGF).[22] Other studies suggest the role of MMPs in HT after stroke because of their roles

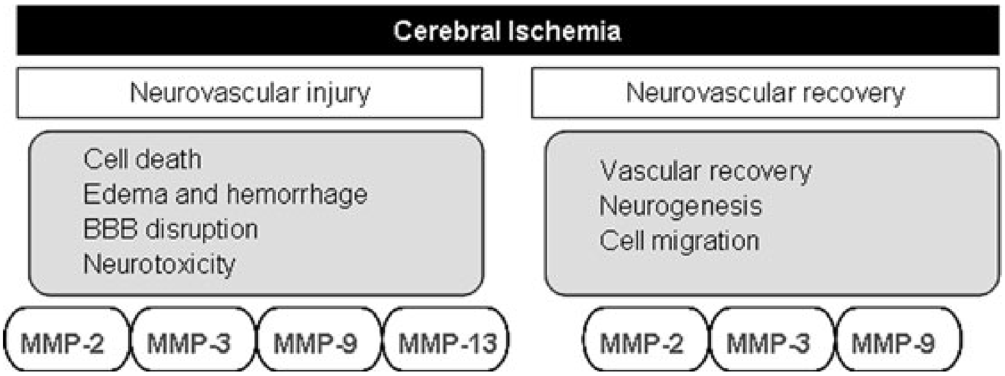

Figure 1. MMP's biphasic role in animal cerebral ischemia: implication in brain injury and recovery after stroke.

in basal lamina degradation and subsequent BBB leakage. An elevation in MMP-2 has been observed in transient focal cerebral ischemia in the early stages of the injury. Claudin-5, one of the proteins found in the tight junctions, is degraded by MMP-2, leading to BBB opening.[23,24] On the other hand, BBB leakage was reduced in MMP-9 but not MMP-2 knockout mice.[19,25,26] In addition to vascular leakage, extracellular matrix proteases may also directly induce cell death by disrupting homeostatic signals between cells and matrix.[27] In this regard, the importance and relevance of these matrix mechanisms has been highlighted by the finding that fibronectin knockout mice suffered increased neuronal apoptosis and brain infarction after focal cerebral ischemia.[28]

Although the investigations have been focused on MMP-2 and MMP-9 roles, other MMP members may play important roles as well. For example, MMP-3 can be activated after ischemia-reperfusion in rat brain, causing the cleavage of the cerebral matrix agrin and contributing to BBB opening during neuroinflammation after intracerebral lipopolysaccharide (LPS) injection in mice.[29,30] Another investigation suggested a role for MMP-3 in the intracranial bleeding that occurs after thrombotic middle cerebral artery occlusion in mice.[31] The authors showed that MMP-3 expression was significantly elevated in ischemic tissue, and MMP-3 knockout mice treated with t-PA had significantly reduced HT than t-PA-treated WT mice. Finally, MMP-13 was recently found to be upregulated in rat brain after ischemia and localized in the nucleus of neural cells.[32] Other studies found that the expression of MMP-13 was increased in the BBB-damaged hippocampal vessels in stroke-prone spontaneously hypertensive rats.[33]

Interestingly, relevant data has been published in human studies (Table 1). Two early studies published in 1997 demonstrated for the first time that MMP-9 and MMP-2 are elevated in the ischemic human brain.[34,35] More recent data confirmed the presence of high MMP-9 levels not only in infarcted tissue, but also in the peri-infarct areas, suggesting a role for MMPs in the process of infarct growth.[16] Similar findings have also been reported for perihematoma tissue from patients that suffered from hemorrhagic stroke.[16] Importantly, MMP-9 levels seem to peak within infarcts that undergo hemorrhagic conversion, correlating with enhanced erythrocyte extravasation and neutrophil infiltration surrounding the affected capillaries, together with severe collagen IV degradation in the surrounding basal lamina.[36] Recently, increased MMP-13 expression was observed in infarct and peri-infarct brain samples, suggesting that they were mainly produced by neurons with an intranuclear localization.[32] These human studies are consistent with animal model data showing microvascular basal lamina injury and loss of collagen type IV, which can be reversed with hypothermic treatments that reduce enzymatic activity of MMP-2 and MMP-9 in ischemia-reperfusion rat models.[37–38]

After stroke, it is attractive to hypothesize that decoupling of this putative MMP network may occur. Future studies may be required to assess not just MMP-2, -3, -9, or -13 but all family members to truly "fingerprint" the role that the proteases play in acute neurovascular injury. With this global screening concept, a recent study in human brain samples has combined laser microdissection techniques with protein arrays to elucidate the cell-specific expression of the MMP family. In this study, neurons and

Table 1. MMPs as biomarkers in human stroke

MMP	Response	References
MMP-1	Upregulated in infarcted tissue	39
MMP-2	Elevated 2–5 first days after stroke Elevated after ICH Upregulated in infarcted tissue Discrepancies about relation with infarct growth and neurological status	15, 16, 34, 35, 37–40, 42, 47, 59, 60
MMP-3	Related with mortality in ICH Elevated after ICH Upregulated in infarcted tissue	39, 40
MMP-8	Upregulated in infarcted tissue	39
MMP-9	Elevated in ischemic stroke Related with infarct growth Upregulated in infarcted tissue Related with poor neurological outcome Increases risk of HT Upregulated after t-PA therapy Elevated after ICH Related with peri-hematoma edema in ICH	15, 34–42, 45–47, 55, 56, 59–61
MMP-10	Upregulated in infarcted tissue	39
MMP-13	Upregulated in infarcted and peri-infarcted tissue Discrepancies about prediction of infarct growth	32, 61, 62

endothelial cells were isolated from both ischemic and contralateral brain tissue. The protein array revealed that globally MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-13 and TIMP-1 were upregulated in the infarcted tissue, whereas the subcellular study showed that MMP-9 and TIMP-2 were highly produced in brain microvessels. Also, this array demonstrated that neurons were responsible for MMP-10 secretion in the ischemic brain but not in healthy areas.[39]

Although less frequent, hemorrhagic stroke is a devastating cerebrovascular event with high rates of mortality. Several studies suggest that similar elevations in MMP-2, -3, and -9 along with imbalances in TIMPs are indeed present after intracranial intracerebral hemorrhage.[40] However, the temporal profile of these responses may be quite different. In this study and another, MMP-9 was found to be related to peri-hematomal edema volume and neurological worsening, whereas MMP-3 was strongly related with mortality.[40,41] Another group investigated subarachnoid hemorrhage and found that MMP-2 levels were decreased, whereas MMP-9 levels were increased in comparison to control subjects.[47]

Matrix metalloproteinases and thrombolysis

Intravenous recombinant tissue plasminogen activator (t-PA) administered within 4.5 h from symptom onset has been proven to be an effective therapy for acute ischemic stroke.[43,44] The most feared complication of thrombolytic therapy is symptomatic intracranial hemorrhage (SICH). To prevent the risk of SICH, only a small fraction of potentially eligible stroke patients are treated with t-PA. When the preceding ischemia is severe and long enough to cause irreversible cellular damage, reperfusion contributes to the occurrence of severe brain injury. Reoxygenation after ischemia mediates the destruction of microvascular integrity followed by capillary occlusion, serum extravasation, and HT.[12]

Elevation of some MMPs within the neuroinflammatory response after stroke has been considered a possible trigger for these hemorrhagic complications. In this regard, MMP-9 levels are elevated in patients with acute ischemic stroke, and the degree of elevation predicts cerebral ischemic HT.[15,46,47] More importantly, it has been shown that MMP-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis and that patients with SICH display a higher baseline MMP-9 level compared with patients with nonsymptomatic hemorrhages or without HTs.[48]

To clarify this t-PA–MMP-9 relation, recent *in vitro* and *in vivo* data suggested that t-PA upregulates MMP-9 after cerebral ischemia. In this regard, Wang and colleagues demonstrated a novel low-density lipoprotein receptor-related protein (LRP)-mediated pathway that implicate t-PA in the direct activation of MMP-9;[49] this report adds new mechanisms of MMP-9 activation to others such as ROS, NO, and mitogen-activated protein (MAP) kinase that are upstream regulators of MMP-9[50–52] and to plasmin that activates multiple MMPs, which may finally activate MMP-9.[53] It is also suggested that neutrophils might be the main source of MMP-9 following t-PA and, therefore, partially responsible for thrombolysis-related HT. In this sense, it has been shown that neutrophil infiltration highly contributes to enhance MMP-9 in the ischemic brain by releasing MMP-9 proform, which might participate in the tissular inflammatory reaction.[54] More recent data has shown that t-PA promotes the release MMP-9, MMP-8, and TIMP-2 and degranulation in human neutrophils.[55]

In vivo studies using rat and mouse models of focal cerebral ischemia have investigated the relationship between t-PA and MMP-9 *in vivo*. Administration of exogenous t-PA increased the MMP-9 response after ischemia in rats. t-PA gene knockout significantly decreased ischemic MMP-9 levels compared with WT mice and exogenous t-PA reinstated the MMP-9 response back up to WT levels. Together, these pharmacological and genetic data show that t-PA can amplify MMP-9 response stroke.[56]

In human patients there is also an association between t-PA and MMP-9 levels. Thrombolytic therapy independently predicted hyperacute MMP-9 level in plasma of acute ischemic stroke patients.[57] More recently, another study has shown again that MMP-9 increases after t-PA administration and that this was lowered if patients received additionally uric acid.[58]

Altogether the present data obtained from both human and experimental studies suggest that selectively blocking MMP-9 during thrombolysis might be a strategy to reduce hemorrhagic complications. In this regard, a recent study in spontaneously hypertensive rats receiving delayed t-PA (6 h) and minocycline showed a reduction in the infarct size, hemorrhage extension, and mortality rates similar to those obtained if t-PA was given shortly after the ischemic event (1 h).[59] This improvement was associated to a strong reduction on MMP-9 when minocycline was administered before delayed t-PA.

Matrix metalloproteinases and infarct size

To date, MMP-9 has been the most studied MMP in relation to stroke. As mentioned before, high levels of this protease have been found in patients with ischemic and hemorrhagic strokes, compared with healthy individuals.[15,46] More importantly, acute MMP-9 levels have been related, besides HT, to infarct size and poor neurological outcome.[15,46,60] MMP-9 levels assessed at hospital entry have been identified as predictors of the infarct volume measured with diffusion-weighted MRI, and this biomarker was further correlated with stroke lesion growth, even with the application of thrombolytic therapy.[61,62] For other MMP family members, the results have been more variable. While some investigations have documented high MMP-2 serum levels after ischemic stroke, others have reported decreased MMP-2 levels compared with nonstroke controls.[15,64] And in some studies, MMP-2 did not appear to have a consistent correlation with neurological status, subtype of hemorrhage, or infarct growth.[15,48,63] Similar variations have been reported for MMP-13. Some researchers have proposed that MMP-13 is an independent predictor of infarct growth at 24 h after stroke onset.[63] But others could not detect clear differences in MMP-13 blood levels 1 day after stroke compared with controls.[65] Regarding TIMPs, measurements of various members of this family (including TIMP-1 and TIMP-2) in blood suggest that imbalances between protease and inhibitor might be present after stroke.[47,60,64]

Blocking MMPs

Blocking the effect of MMPs could prevent their pathologic role. In this way, MMP inhibitors have been used in animal studies to reduce infarct size, apoptosis, and BBB injury (Table 2). BB-94, BB-1101, GM6001, and FN-439 are broad-spectrum MMP inhibitors; they block expression or activation

Table 2. Pharmacological inhibitors of MMPs in experimental studies

Drug	Action	Effects	References
BB-94 (Batimastat)	Broad spectrum MMP inhibitor	Reduces ischemic lesion size Reduces t-PA-induced hemorrhage Blocks BBB opening Decreases delayed neurovascular remodeling	19, 63–65, 71, 72
BB-1101	Broad spectrum MMP inhibitor	Reduces BBB opening at 3h Reduces brain edema at 24h No effect and worse neurological score at 48 h	66–68
GM6001 (Ilomastat)	MMP (collagenase) inhibitor	Reduces ischemic lesion size Reduces t-PA-induced hemorrhage Decreases delayed neurovascular remodeling	31, 69–70
FN-439	Broad spectrum MMP inhibitor	Decreases delayed neurovascular remodeling	71, 72
SB-3CT	MMP (gelatinase) inhibitor	Rescues neurons from apoptosis	73
Antibody clone 6-6B	MMP-9 monoclonal antibody	Reduces ischemic lesion size	20
Statins	HMG-CoA reductase inhibitors	Reduce ischemic lesion size	74–75
Indomethacin	COX inhibitor	Reduces BBB injury Reduces brain edema	77
Trandolapril	ACE inhibitor	Reduces ischemic lesion size Improves neurological outcome	78–79
Diphenylene iodonium	Inhibitor of NADPH oxidase	Reduce BBB injury Improve neurological outcome	80
Zileuton	5-LOX inhibitor	Reduces brain edema Reduces ischemic lesion size	81
Minocycline	Tetracycline antibiotic	Reduces ischemic lesion size Reduces t-PA-induced hemorrhagic events	58, 82, 83

of all MMPs. Batimastat (BB-94) significantly reduced ischemic lesion size in mice but failed to have the same protective effect in MMP-9 knockout mice, demonstrating that MMP-9 plays a deleterious role in the development of brain injury after focal ischemia.[19] BB-94 also attenuates mechanisms involved in t-PA-induced hemorrhage, blocking the opening of the BBB and reducing hemorrhage in rabbits and rats.[64–66] Treatment with BB-1101, another synthetic inhibitor, reduced BBB opening at 3 h and brain edema at 24 h, but neither was affected at 48 h, suggesting that BB-1101 blocked the MMP-9 but not the MMP-2 levels associated with the delayed opening.[67] Inhibition of MMP-9 by BB-1101 reduced occludin protein loss in ischemic microvessels, attenuating early BBB disruption.[68] Other studies added that BB-1101 significantly reduced BBB permeability at 3 h but failed to reduce lesion size at 48 h and impaired the recovery, as shown by a worse neurological score in treated rats.[69] Administration of a broad-spectrum, highly specific MMP inhibitor, GM6001, but not its negative control results in a significant (50%) reduction in ischemic brain volume after focal ischemia in rats.[70] GM6001 also reduced intracranial bleeding after t-PA treatment in WT mice but not in MMP-3 knockout mice.[31]

However, the treatment with GM6001 in mice significantly decreases the migration of neuroblasts from the subventricular zone to the striatum, preventing the neurogenesis potentially impairing neurorecovery.[71] Similar results were found in rats, where the delayed treatment with broad spectrum inhibitors such as FN 439 or BB 94 increased ischemic brain injury, suppressed neurovascular remodeling, and impaired functional recovery from 7 to 14 days after stroke.[72–74] Other authors have investigated the possibility to more specifically block the action of some MMPs. Treatment with SB 3CT, a highly specific inhibitor of gelatinases (MMP 2 and MMP 9), blocked MMP 9 mediated laminin cleavage, thus rescuing neurons from apoptosis.[71] Another strategy to block MMPs is intravenous treatment with specific neutralizing monoclonal antibodies. In a rat model of focal cerebral ischemia, the administration of a monoclonal antibody directed against MMP 9 1 hour before MCAO has shown a significant reduction of infarct size.[69]

In addition to specific MMP inhibitors, there are other drugs that block the effect of MMPs by targeting different pathways (e.g., inflammation). Statins, the most widely used lipid lowering drugs, have been demonstrated to play neuroprotective roles and be clinically significant among neurodegenerative diseases such as vascular dementia, Alzheimer's disease, and Parkinson's disease, as well in cerebral ischemic stroke. The mechanisms of statins in these neurodegenerative disease still partially remain unknown. Several studies in animal models of cerebral ischemia have shown that simvastatin reduces infarct size when injected for 3 days, either before or after induction of ischemia, establishing the therapeutic window of simvastatin. This occurs when the drug exhibits a protective effect, which occurs after a single dose and when the drug is administered within 3 6 h after ischemia.[75] *In vitro* studies using rat astrocytes exposed to t PA treatment demonstrate that the t PA induced MMP 9 secretion was inhibited when simvastatin was present in the culture media. A slight effect was also seen for MMP 2 secretion.[76] In stroke patients, the effect of simvastatin on MMPs has been demonstrated; for example, simvastatin treatment within the first 24 h of the ischemic event prevented the elevated MMP 9/TIMP 1 serum ratio at day 7.[77] Therefore, the present data suggests that acute statin treatment may be beneficial in ischemic stroke through their anti inflammatory actions.

Cyclooxygenase (COX) inhibitors have also been studied for their relationship to TNF α, which is involved in BBB injury and brain edema through upregulation of MMPs. Indomethacin, an anti inflammatory drug and a nonselective inhibitor of COX 1 and COX 2, significantly attenuated MMP 9 and MMP 3 expression and activation and prevented the loss of endogenous radical scavenging capacity following intracerebral injection of TNF α in rats.[78] Tandrolapril, an angiotensin converting enzyme (ACE) inhibitor, reduced infarct size and neurological severity scores in rats after middle cerebral artery occlusion by reducing both MMP 9 and MMP 2 activities in treated animals.[79,80] MMP 2 and MMP 9 activity was also reduced by diphenyleneiodonium (DPI), an inhibitor of the NAD(P)H oxidase, when administered together with dimethylsulfoxide (DMSO). This attenuated postischemic BBB damage and improved neurological outcome after ischemia reperfusion injury in rats.[81] 5 lipoxygenase (5 LOX) is another enzyme activated after cerebral ischemia and related with MMP 9 levels. The selective 5 LOX inhibitor Zileuton downregulated MMP 9 expression and reduced cerebral water content and infarct volume at 24 h after permanent middle cerebral artery occlusion in rats.[82] Finally minocycline, a lipophilic tetracycline antibiotic recognized to have antiapoptotic and anti inflammatory properties, has also been widely studied as a possible neuroprotective agent in several models of brain injury and has had promising results. Administration of minocycline in rats and mice significantly reduced gelatinolytic activity and concentration of ischemia elevated MMP 2 and MMP 9 and reduced infarct size in WT but not MMP 9 deficient mice.[83,84] Moreover, as mentioned before, a recent study has demonstrated its ability to reduce t PA induced hemorrhagic complications and mortality rates if given before thrombolytic treatment strongly associated to a clear suppression of MMP 9 brain content.[49]

Matrix metalloproteinases and neurovascular repair after stroke

We have just reviewed studies supporting the idea that deregulation of MMPs may underlie tissue damage during acute stroke and in part explain some of the complications of thrombolytic therapy. But we have also highlighted that several studies have pointed out that these neurovascular proteases might contribute to beneficial remodeling during stroke recovery (Fig. 1). Then, caution must be exercised when planning to block these proteases.

In this regard, it is known that MMPs are expressed during development and contribute to morphogenesis of the CNS.[85] MMPs may also modulate bioavailable levels of various growth factors by processing proform precursors or by liberating active molecules from matrix-hidden compartments. For example, the ability of MMP-9 to mobilize VEGF from the ECM can activate quiescent vasculature, thus switching on the vascular system to ramp up angiogenesis in both normal and neoplasic tissues.[86] Other authors have demonstrated roles in regeneration for MMPs in the injured CNS. A discrete expression of some MMPs can have beneficial roles in remyelination.[87] In a lysolecithin-induced demyelination toxic model, MMP-9 knockout mice were impaired in myelin reformation.[87] The corresponding rescue experiment demonstrated that MMP-9 expressed locally around a demyelinating lesion of the spinal cord facilitated remyelination. While acute MMP inhibition improved locomotor recovery, extended treatment failed, consistent with the idea that delayed remodeling requires MMP activity in the CNS.

During neurorecovery, increased neurogenesis can be triggered by CNS insults such as stroke or trauma.[88,89] After stroke, newly born neuroblasts migrate from the SVZ to peri-infarct cortex, and increased vascular remodeling is also found in this area.[90,91] Mice deficient in MMP-9 show continued demyelination after injury, perhaps because of a failure to clear injury-induced deposits of the NG2 proteoglycan.[87] At 2 weeks after stroke in mice, MMP-9 was enhanced in the SVZ and colocalized with BrdU-labeled cells and neuroblasts.[71] Furthermore, inhibition of MMPs reduced the extension of neuroblast signals that extended from the SVZ into the damaged striatum. Other proteases, such as MMP-3, also participated in neurogenesis, and together with MMP-9 mediated the differentiation and chemokine-induced cell migration of adult neural/stem progenitor cells.[92] These data indicate that MMPs may contribute to endogenous repair mechanisms by assisting in the migration of neuroblasts after stroke.

Conclusions

Extensive data from molecular, cellular, animal models, and human studies support a deleterious role for MMPs in acute stroke and their relation with infarct size, brain edema, or HT events. Nevertheless, MMPs play complex and multiple roles after stroke and participate in neurovascular remodeling during delayed phases of stroke recovery. Several types of MMP inhibitors, including anti-inflammatory drugs, have been used in a number of animal studies showing its potential benefits when given in the acute phase of stroke but perhaps compromising stroke recovery in delayed phases. Then, pharmacologic targeting will have to optimize acute inhibition of deleterious MMP actions without compromising the beneficial effects of matrix plasticity during stroke recovery.

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Conflicts of interest

Authors declare no conflicts of interest.

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