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Adhesion-mediated signal transduction in human articular chondrocytes: the influence of biomaterial chemistry and tenascin-C

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Abstract

Chondrocyte 'dedifferentiation' involves the switching of the cell phenotype to one that no longer secretes extracellular matrix found in normal cartilage and occurs frequently during chondrocyte expansion in culture. It is also characterized by the differential expression of receptors and intracellular proteins that are involved in signal transduction pathways, including those associated with cell shape and actin microfilament organization. The objective of this study was to examine the modulation of chondrocyte phenotype by cultivation on polymer substrates containing poly(ethylene glycol) (PEG). We observed differential arrangement of actin organization in articular chondrocytes, depending on PEG length. When cultivated on 300 g/mol PEG substrates at day 19, chondrocytes had lost intracellular markers characteristic of the differentiated phenotype, including type II collagen and protein kinase C (PKC). On these surfaces, chondrocytes also expressed focal adhesion and signaling proteins indicative of cell attachment, spreading, and FA turnover, including RhoA, focal adhesion kinase, and vinculin. The switch to a dedifferentiated chondrocyte phenotype correlated with integrin expression. Conversely, the expression of CD44 receptors coincided with chondrogenic characteristics, suggesting that binding via these receptors could play a role in maintaining the differentiated phenotype on such substrates. These effects can be similar to those of compounds that interfere in intracellular signaling pathways and can be utilized to engineer cellular response.

Introduction

The characteristic phenotype of differentiated chondrocytes is that of rounded cells that secrete extracellular matrix proteins (specifically collagen II and aggrecan) and with a diffuse actin microfilament network [1,2]. Upon attachment to substrates in two dimensions however, chondrocytes have frequently been observed to attain a spread morphology with a reorganization of filamentous actin into distinct stress fibers [3–5]. During this dedifferentiation towards a more fibroblastic phenotype, type II collagen production is reduced and eventually replaced with type I collagen, with concomitant reduction or cessation of aggrecan synthesis [3,4].

A number of researchers have investigated techniques to reexpress the chondrogenic phenotype during chondrocyte expansion in monolayer culture by growing cells on microcarriers [6] using growth factors, such as basic fibroblast growth factor (bFGF-2) [7,8] or incorporating cytoskeleton modifying drugs such as cytochalasin D or dihydrocytochalasin B [5,9–16]. And although there have been reports discussing cell behavior on different substrates [17–21], there has been little in the way of mechanistically addressing the influence of materials on the events that regulate cellular phenotype. Understanding such behavior

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has become increasingly relevant, not only for investigative cell science but because of the possibilities for cell transplantation and tissue engineering using polymers as cell delivery devices [22].

Given the importance of understanding cell response to biomaterials, we have attempted to answer the following question: How do substrate properties influence cell behavior? In addition, what substrates elicit the expression of intracellular signaling proteins that are involved in the maintenance of the differentiated chondrogenic phenotype?

To that end, we used block copolymers of poly(ethylene glycol) terephthalate (PEGT) and poly(butylene terephthalate) (PBT) as model substrates for cell attachment and growth. The overall copolymer properties are determined by its two components—the PEG segment is hydrated, whereas PBT provides hydrophobic, protein binding domains, and stiffness. These copolymers can be synthesized with different fractions of soft and hard components, as well as with PEG chains of different molecular weights. These were shown to be biocompatible [23–25] and have been studied extensively for tissue engineering applications [25–28]. The ability to synthesize polymers with controllable properties makes them useful model substrates for investigating cell behavior.

Focal adhesions (FAs) are the primary sites by which cells detect their substrate. They are plasma membrane clusters of receptors, cytoskeletal proteins, and other proteins that link the transmembrane receptors to the cytoskeleton [29,30]. They form the foci of signal transduction from the external substrate-dependent microenvironment to cells (outside–in signaling), as well as of feedback signals transmitted by cells to their external milieu (inside–out) [31–33]. It has also been suggested that FAs are sites where mechanical stress is converted to biochemical signals [34,35]. Thus, the signaling mechanisms in play at, or due to FAs, are crucial in determining cell fate, especially as it relates to differentiation or proliferation. Given the complexities of intracellular signaling and cross-talk between pathways, we have used a candidate approach to examine the expression of selected proteins that are known to be involved in general FA formation and turnover, as well as those associated with the maintenance of differentiated chondrogenic characteristics.

We also studied the effects of blocking the activation of RhoA by tenascin-C (TN). RhoA is involved in cytoskeletal regulation [36,37], a feature we exploited to probe how interference in RhoA signaling by using soluble TN (an exogenous matrix protein) or varying substrate chemistry can affect stress fiber assembly and FA formation, thereby modulating chondrocyte differentiation.

The objective of this study was to examine the differential expression of FA receptors and intracellular signaling proteins involved in preserving the chondrogenic phenotype, after varying polymer substrate composition and by using an exogenous compound to interfere in cell signaling.

Materials and methods

Polymer nomenclature

The different formulations of this copolymer system are indicated as follows: *a*-PEG *b*:*c*, where *a* is the molecular weight of PEG (g/mol), *b* is the weight percentage of PEGT, and *c* is the weight percentage of PBT. For example, the polymer 1000-PEG 70:30 has PEG molecular weight of 1000 g/mol and a PEGT–PBT ratio of 70:30.

Processing of polymer culture substrates

Polymer particles were dissolved in chloroform (Sigma, Uithoorn, The Netherlands) and 60–100 μm thick dense films were cast on a glass plate. These substrates were vacuum dried for 2 days, gamma sterilized, and immersed overnight in complete medium before cell seeding.

Cell isolation and culture

Articular cartilage was harvested from the resected femora of female patients, pursuant to institutional guidelines of informed consent. Primary chondrocytes were isolated by type II collagenase (Worthington Biochemical, Lakewood, NJ) digestion for 20 h, rinsed in phosphate buffered saline (PBS) containing $CaCl_2$, and $MgCl_2$ at pH 7.4 (Invitrogen, Breda, The Netherlands) with 15% fetal bovine serum (FBS) (Invitrogen) and transferred to a well-defined culture medium [Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% FBS, 1 mM sodium pyruvate (Invitrogen), 50 μg/ml penicillin, 50 μg/ml streptomycin, 0.4 mM L-proline, 0.1 mM nonessential amino acids, and 10 mM HEPES buffer (Sigma)].

Once isolated, chondrocytes were seeded at a density of 10,000 cells/cm^2 on PEGT-PBT based polymer films and on presterilized tissue culture polystyrene (TCPS) cover slip controls (Falcon). Cells were harvested 19 days after seeding. The experiments were performed in triplicate.

Western blotting

Cells were lysed by boiling in standard Laemmli sample buffer for 10 min. Aliquots of cell lysates were adjusted to ensure the loading of equal amounts of total proteins into wells of SDS–polyacrylamide gels by RC-DC assay (Bio-Rad, Veenendaal, The Netherlands). Samples were electrophoresed at 120 V. Decasted gels were soaked in blot buffer for 15 min and the blots transferred to an

Immobilon-P membrane (Millipore, Amsterdam, The Netherlands) by blotting for 1 h at 60 V, 120 mA. Blots were blocked by 30 min incubation in PBS/0.5% Tween X-100 (Sigma)/2% gelatin (CalBiochem, San Diego, CA). This was followed by 1 h incubation at RT with each of the primary antibodies. The primary antibodies used were anti-human vinculin monoclonal antibody (mAb) hVIN-1 (Sigma), anti-human phosphorylated focal adhesion kinase (p-FAK) mAb 14 (BD Biosciences, San Diego, CA), anti-human protein kinase C-α (PKC-α) from the PKC sampler kit (BD Biosciences), and anti-human RhoA mAb 55 (BD Biosciences). Blots were rinsed 3 × 10 min in PBS/0.5% Tween X-100 and incubated 1 h at RT in PBS/0.5% Tween X-100/2% gelatin with the appropriate AP-conjugated secondary antibodies (Sigma), followed by rinsing 3 × 10 min in PBS/0.5% Tween X-100. An AP conjugate substrate kit (Bio-Rad) was used for the colorimetric detection of proteins.

Localization of actin, type II collagen, and focal adhesion proteins

At day 19, cells were examined for actin by fixing in 4% paraformaldehyde (Sigma) for 15 min at RT, rinsing twice in PBS (Invitrogen), permeabilizing with 0.1% Triton X-100 in PBS for 5 min at RT, blocking for 15 min with PBS/0.1% glycine (Sigma), and incubating with Alexa Fluor-labeled phalloidin (Leiden, The Netherlands) for 5 min at RT. TCPS discs and PEGT/PBT substrates with adhered cells were mounted between glass slides and coverslips with a medium containing DAPI (Vector Labs, Burlingame, CA). To observe the localization of FA components, cells were fixed for 15 min with 4% paraformaldehyde, rinsed with PBS, and blocked for 30 min with serum-free protein block (DAKO, Glostrup, Denmark). Cells were further rinsed with PBS and incubated separately with each of the following monoclonal antibodies: anti-human type II collagen (Rockland, Gilbertsville, PA), dilution 1:100; anti-human vinculin mAb hVIN-1 (Sigma), dilution 1:400; anti-α5β1 P1D6 (Covance, Princeton, NJ), dilution 1:500; anti-αvβ3 VI-PL2 (Pharmingen, San Diego, CA), dilution 1:100; anti-CD44/FITC BU52 (Ancell, Bayport, MN), dilution 1:100. Cells were rinsed in PBS and further incubated for 30 min with appropriate Alexa Fluor-conjugated secondary antibodies (Molecular Probes) except for the FITC-conjugated anti-CD44 mAb. Cells were further washed three times with PBS before mounting with an antifading medium (Vector Labs). All fluorescent images were obtained with a Nikon microscope (E600) equipped with a multiband filter (Omega Opticals, Brattleboro, VT). Images were acquired by a Nikon digital camera.

Cytoskeletal modification by tenascin-C

To test the effects of the RhoA inhibitor tenascin-C (TN) on chondrocyte attachment, spreading, and actin structure, human articular chondrocytes were expanded in flasks and upon reaching confluence after two passages were trypsinized and resuspended in culture medium as defined previously. Cells were supplemented with either 35 or 70 nM TN (Chemicon, Temecula, CA). A TN-free group was also used as the negative control. TN was added to culture medium before replating since we found that once attached to their substrates, chondrocyte sensitivity to exogenous TN was reduced. The p3 chondrocytes were then replated (n = 5) on TPCS slides. Samples were harvested at 1.5, 5, and 24 h and stained for actin (phalloidin) and nuclei (DAPI) as described above. Chondrocytes harvested at 24 h (−TN, +70 nM TN) were also assayed for Rho by Western blotting as described above.

A list of frequently used abbreviations in this paper is given in Table 1.

Results

Actin cytoskeleton arrangement and modification

Inhibition of RhoA expression and modulation of cell shape and actin stress fiber organization by tenascin-C

Cellular actin distribution was observed by binding to fluorescently labeled phalloidin. There was a time and dose-dependent inhibition of cell spreading by soluble Tenascin-C (Fig. 1). When cultured on TCPS for 5 h, p3 chondrocytes were spread in −TN medium, thinly elongated in 35 nM TN conditions, and completely rounded with diffuse actin that was concentrated towards the cell membrane in 70 nM TN (Figs. 1a–c). By 24 h, cells had spread further in the control

Table 1
A list of abbreviations frequently used in this paper

Abbreviation	Description	Abbreviation	Description
PEGT	poly(ethylene glycol) terephthalate	FGF	fibroblast growth factor
PBT	poly(butylene terephthalate)	PKC	protein kinase C
FA	focal adhesion	TCPS	tissue culture polystyrene
p-FAK	phosphorylated focal adhesion kinase	TN	tenascin-C
MF	microfilaments	ECM	extracellular matrix
IF	intermediate filaments	MT	microtubules

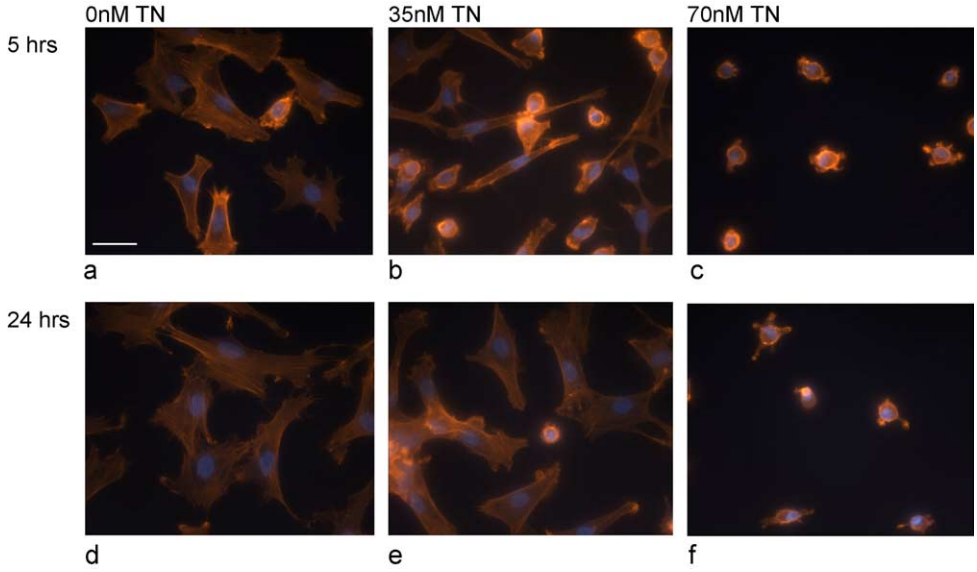

Fig. 1. Chondrocytes attached to TPCS slides with soluble TN at different concentrations at 5 and 24 h after adding TN. When 70 nM TN was used, the attached cells remained rounded at both time points. The scale bar in panel a is 20 μm. All images were obtained at the same magnification.

(TN-free) and 30 nM TN conditions but remained rounded in 70 nM TN (Figs. 1d–f).

RhoA expression

Western blotting for RhoA revealed substrate-dependent expression of this actin cytoskeleton-regulating GTPase (Fig. 2). Interestingly, RhoA expression was inhibited by primary chondrocyte attachment to 1000 g/mol PEG substrates, as well as by the inclusion of 70 nM TN with p3 chondrocytes.

Actin organization

At day 19, chondrocytes were rounded and a diffuse actin network was observed within cells cultivated on polymers with 1000 g/mol PEG, with a cortical actin structure predominantly just inside the cell membrane (Fig. 3a). On 300 g/mol PEG polymers, however, chondrocytes exhibited a spread morphology with distinct actin stress fibers (Fig. 3b). These actin cables were observed on 300 g/mol polymers regardless of PEGT–PBT ratio (data not shown).

Expression of type II collagen, focal adhesion components and chondrogenic signaling proteins is substrate dependent

Type II collagen is a characteristic matrix protein secreted by differentiated chondrocytes in hyaline cartilage. Immunofluorescence analysis revealed that on substrates with 1000 g/mol PEG, type II collagen was expressed in the pericellular environment of rounded chondrocytes (Fig. 4a). Conversely, this was not expressed in chondrocyte colonies on 300 g/mol surfaces (Fig. 4b).

The focal adhesion protein vinculin serves as a protein linking integrins and the cytoskeleton; its expression was examined by immunofluorescence microscopy and Western blotting.

Vinculin—immunofluorescence labeling at day 19

Vinculin was concentrated towards the cell periphery of chondrocytes cultured on 1000 g/mol PEG polymers, whereas it was found to be distributed across the surfaces of chondrocytes on 300 g/mol PEG substrata (Figs. 5a and b).

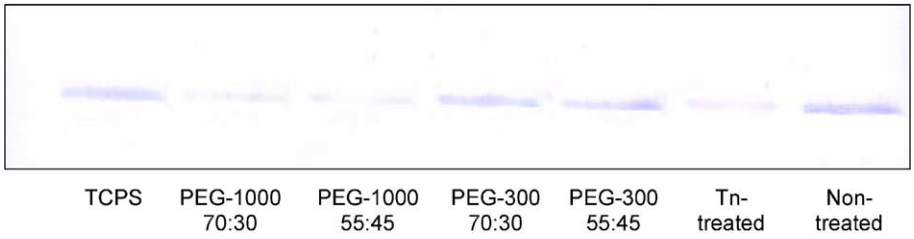

Fig. 2. Western blot for RhoA GTPase expression by primary chondrocytes cultivated on polymer compositions, as well as by p3 chondrocytes in the presence or absence of tenascin-C. RhoA expression was found to be substrate dependent and its inhibition on 1000 g/mol polymer substrates was similar to that achieved by the inclusion of 70 nM tenascin-C to the cells.

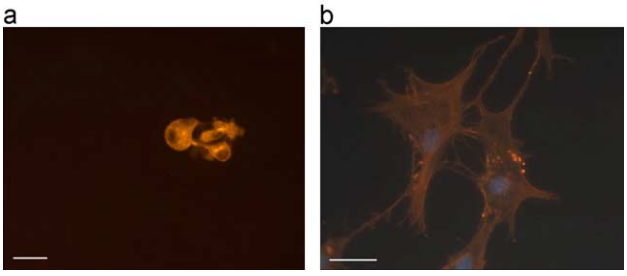

Fig. 3. Chondrocyte actin network visualized by fluorescently conjugated phalloidin. Cells were cultivated for 19 days on 1000-PEG 70:30 (a) and 300-PEG 55:45 (b) substrates. Other than a more spread morphology, actin was organized into more distinct stress fibers when attached to 300-PEG 55:45 polymers. Scale bars in panels a and b are 20 and 10 μm, respectively.

Vinculin—Western blots

Given the role of vinculin in linking FAs to the actin cytoskeleton, we evaluated relative vinculin expression on each of the different culture substrates by Western blotting (Fig. 5c). Relative vinculin expression on the different polymer compositions was found to be as follows (in descending order): TCPS, PEG-300 55:45, 300-PEG 70:30, 1000-PEG 55:45, 1000-PEG 70:30.

Expression of α5β1 and αvβ3 integrins

Immunofluorescence examination at day 19 of the fibronectin receptor α5β1 integrin and vitronectin receptor αvβ3 integrin revealed that both integrins were expressed uniformly at the surfaces of chondrocytes cultured on 300 g/mol PEG polymers (Figs. 6a and b). However, chondrocytes cultured on 1000 g/mol PEG polymers did not express either of these integrins (not shown).

CD44

The proteoglycan receptor CD44 was expressed by chondrocytes cultivated on 1000 g/mol PEG polymers (Fig. 7a) but was negligible in cells grown on 300 g/mol PEG polymers (Fig. 7b).

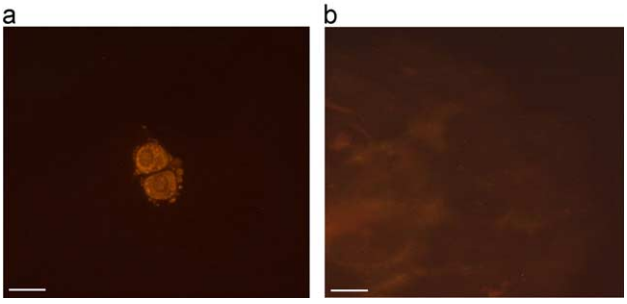

Fig. 4. Immunofluorescence labeling for type II collagen; at 19 days, rounded chondrocytes on 1000-PEG 70:30 substrates were seen to have secreted type II collagen into the pericellular environment (a), characteristic of the differentiated chondrocyte phenotype. Negligible background staining of the fluorescent label was evident on cells cultivated on 300-PEG 55:45 substrates (b). Scale bars = 10 μm.

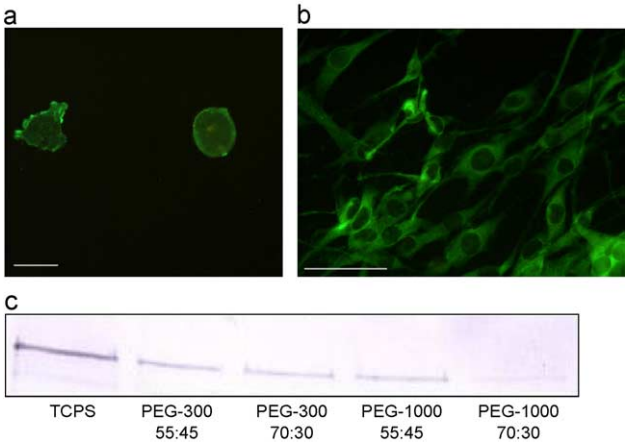

Fig. 5. Immunofluorescence and Western blot labeling for vinculin at chondrocyte–polymer focal adhesions. The scale correspond to 10 μm (a) and 40 μm (b). Little vinculin could be seen by immunofluorescence in chondrocytes on 1000-PEG 70:30 substrates (a), which was found mostly towards the outer edge of the cell membrane, whereas it was distributed across the membrane in chondrocytes cultivated on 300-PEG 55:45 polymers. Western blotting revealed a substrate-dependent expression of vinculin by chondrocytes (c). Samples were normalized to the amount of total protein in cell lysates before loading. Scale bars in panels a and b are 10 and 50 μm, respectively.

Focal adhesion kinase

Activated (phosphorylated) focal adhesion kinase (p-FAK) is an indicator of FA activity and was assayed for by Western blotting (Fig. 8) [38,39]. There was differential composition-dependent expression of p-FAK at day 19. Maximum expression was found in chondrocytes cultured on TCPS, followed sequentially (in descending order) by PEG-300 55:45 and PEG-300 70:30. p-FAK expression was not enough to be detected in chondrocytes that had been cultivated on 1000 g/mol PEG substrates. This demonstrated that FA turnover was maximal on TPCS and 300 g/mol PEG substrates, as also evidenced by enhanced cell spreading and philapodia.

Protein kinase C (PKC)

The PKC-α isoform of PKC has been shown to be an intracellular signaling marker that is expressed during the

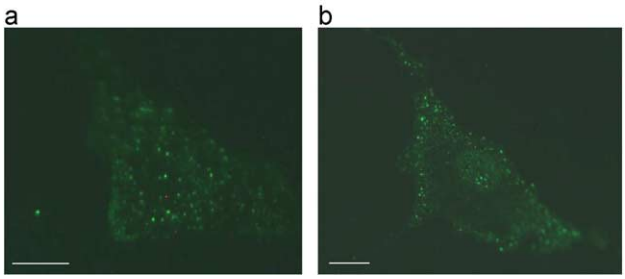

Fig. 6. Immunofluorescence labeling of the expression of α5β1 integrin (a) and αvβ3 integrin (b) by chondrocytes on 300-PEG 55:45 polymer substrates. Chondrocytes cultivated on PEG-1000 70:30 did not express either of these integrins. The scale bar in each image corresponds to 10 μm.

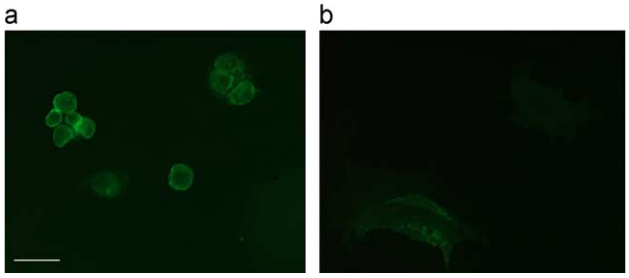

Fig. 7. The proteoglycan receptor CD44 visualized by immunofluorescence labeling of chondrocytes cultivated on 1000-PEG 70:30 (a) and 300-PEG 55:45 (b) substrates. The rounded cells on PEG-1000 70:30 polymers demonstrated a higher intensity of peripheral staining, whereas only minimal CD44 expression could be detected in chondrocytes attached to 300-PEG 55:45 substrates. The scale bar in panel a corresponds to 10 μm, both images are at the same magnification.

redifferentiation of chondrocytes in three-dimensional culture conditions after serial expansion, as well as during induced chondrogenesis of mesenchymal condensations [40,41]. Western blot analysis of PKC-α revealed expression only in chondrocytes cultivated on 1000 g/mol PEG polymers for 19 days, with no detectable expression when grown on 300 g/mol PEG substrates or TCPS (Fig. 9).

Discussion

There have been previous reports that examined the effects of substrate chemistry on chondrocyte behavior [27,42–46]. We have recently shown that chondrocyte attachment and phenotypic gene expression did not always correlate with substrate wettability, but with differential protein adsorption from serum [47]. Specifically, the unique affinities of vitronectin and fibronectin for different substrates influenced the modalities of cell attachment and downstream gene expression. In the present study, we extend that analysis to include the intermediary events that occur between the initial protein adsorption to biomaterials and eventual gene expression. The cytoskeletal organization, cell shape, and expression of the actin network-regulator GTPase Rho were also compared to those of adding the RhoA inhibitor, tenascin-C, to the cultures.

Depending largely on the lengths of the substrate PEG molecules, we observed striking differences in chondrocyte shape, actin cytoskeletal arrangement, type II collagen, FA composition, and the expression of the intracellular signaling proteins RhoA, focal adhesion kinase, and protein kinase C. The interactions between the various FA and signaling proteins are complex, and we have attempted to note trends and suggest correlations that can be associated with the modulation of differential chondrocyte phenotype by polymer substrates that are being evaluated for cell delivery.

The involvement of the actin network in regulation of the chondrogenic phenotype has long been known, and distinct actin stress fiber assembly, and not just spread cell morphology, has been shown to correspond to a dedifferentiated fibroblast-like phenotype [5,9–13,16]. Since the early correlations between cellular markers and state of the chondrogenic phenotype were based on the organization of actin microfilaments (MF), the disruption of actin stress fibers by drugs has been studied as a possible tool by which to induce redifferentiation [5,10,12,16]. The compounds often used are cytochalasin D and dihydrocytochalasin B (DHCB), toxic agents that function by disrupting actin stress fibers and inhibiting de novo actin polymerization by capping the ends of the shredded filaments. Therefore, although they serve a useful purpose in understanding the effects of actin stress fiber disruption, their toxicity is likely to preclude their application in noncancerous orthobiologic repair.

However, to utilize the potential benefits of actin modification in a practical setting, we used tenascin-C (TN), an ECM protein that has been shown to block RhoA activation by integrin-mediated inhibition of tyrosine phosphorylation [48]. Therefore, given the crucial function of RhoA in cytoskeletal regulation [36,37], we used soluble TN to inhibit stress fiber assembly and FA formation, with the expectation that it would result in cytoskeleton-induced redifferentiation. TN did indeed inhibit RhoA expression in a time-dependent manner, and when compared to the effects of biomaterial composition, we found that the inhibition of RhoA by adding soluble TN to p3 human chondrocytes was similar to that in primary chondrocytes cultured on 1000 g/mol PEG polymers. Studies with various cell types have shown that the preservation of cytoskeletal tension is critical for G1 progression and that disrupting actin or inhibiting Rho blocks entry into S-phase [49]. Together with our data, this demonstrates that the manipulation of biomaterial chemistry can allow or block intracellular production of

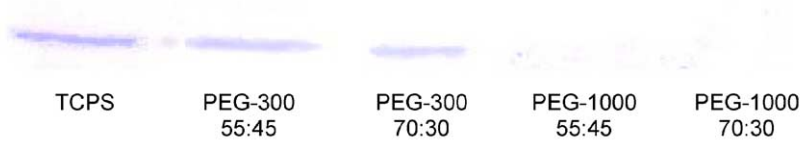

Fig. 8. Western blot for activated (phosphorylated) focal adhesion kinase (p-FAK) expression by chondrocytes cultivated on various polymer compositions for 19 days.

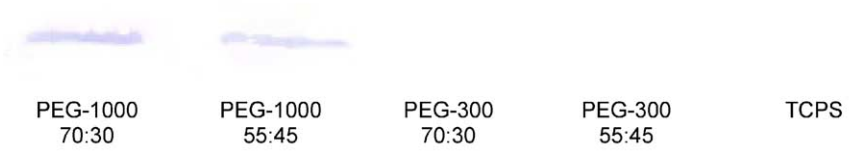

Fig. 9. Western blot for protein kinase C (PKC-α) expression by chondrocytes on various polymer substrates at day 19.

signaling pathway proteins in a manner similar to the inclusion of exogenous pathway inhibitory compounds.

The changes in chondrocyte shape after addition of TN to cells grown on TPCS surfaces revealed dose dependency on soluble TN: 35 nM TN was inadequate to maintain the rounded morphology 24 h after treatment, whereas 70 nM was sufficient. Similar shape, size, and cytoskeletal effects were observed when surfaces were coated with tenascin-C (data not shown). The actin network in chondrocytes cultivated on substrates with longer PEG chains was diffuse and concentrated towards the cell membrane, indicating that the actin organization remained similar to that of differentiated primary chondrocytes as well as chondrocytes in articular tissue in vivo [50]. The actin cytoskeleton plays an important role in regulating phenotype and morphogenesis because many of the proteins involved in transduction pathways are immobilized on the actin MF [51]. Therefore, actin MF disassembly would prevent the binding of these signaling proteins with other moieties, such as enzymes that are responsible for the activation of signaling proteins and the transduction of mechanical cues into biochemical signals.

No direct correlation between the state of the chondrocyte phenotype and either intermediate filaments (IF) or microtubules (MT) has been reported. Our observations also did not reveal differential arrangements of these cytoskeleton components after chondrocyte cultivation on the different PEGT–PBT polymers (data not shown).

These results reinforced the importance of the cytoskeleton and cell morphology in chondrocyte phenotype and function. They also warranted further investigation into the effects of biomaterial substrate chemistry on specific proteins involved in cellular adhesion and chondrocyte phenotype modulation.

Type II collagen is secreted by chondrocytes in normal hyaline cartilage [1,52,53]. This ECM protein is therefore a key marker of the differentiated chondrogenic phenotype. The expression of type II collagen correlated with diffuse actin organization. Conversely, it was not present around chondrocytes with stress fiber formation. Type II collagen data enable more definite observations and comparisons of the phenotypic state of cells on the different substrates.

Vinculin is an FA protein that has been reported to colocalize with actin at cell-substrate focal contacts [54]. Vinculin mediates the anchoring of actin stress fibers to plasma membrane receptors and in stabilizing cell shape [35,55] and is therefore a good indicator of the degree of cell adhesion and spreading. The greater amount of vinculin found by Western blotting (Fig. 5c) to be expressed in chondrocytes on the 300 g/mol PEG polymers was indicative of their transformation to a more fibroblastic phenotype with increased expression of FA components. The differential expression and distribution of vinculin in 300 g/mol PEG copolymer–chondrocyte focal contacts suggested that vinculin expression was involved in signaling, either biochemical, mechanically transduced, or both, which resulted in the loss of chondrogenic phenotype on these substrates. Given the cytoskeleton–FA contact stabilizing role of vinculin, this likely occurred via the assembly of actin stress fibers and the consequent cellular tension.

The expression of both α5β1 and αvβ3 integrins, but absence of the receptor CD44, at the FAs of chondrocytes cultured on 300 g/mol PEG substrates suggested that their attachment was mediated via integrin binding to the Arg-Gly-Asp (RGD) sequences of the adhesive proteins fibronectin and vitronectin [56,57]. However, absence of these integrins but positive expression of CD44 in chondrocytes cultivated on the longer PEG substrates was suggestive of cell attachment via hyaluronan binding regions of the same proteins [58,59]. CD44 is expressed by differentiated chondrocytes in hyaline cartilage and is known to mediate chondrocyte adhesion to sulfated proteoglycans (aggrecan) in normal cartilage ECM, thereby linking matrix proteoglycans to chondrocyte plasma membranes [60,61]. The receptor plays a key role in cartilage homeostasis and its expression is known to correlate with chondrogenesis [62–64]. Therefore, it is not surprising that in vitro, the ability of substrates to maintain or elicit CD44 expression would present the differentiated phenotype. It should be noted, however, that although chondrocytes in vivo do express some α5β1 integrin, this expression is far less than during monolayer culture on TCPS [65]. These data demonstrate the correlation between the expression of α5β1 integrin and chondrocyte dedifferentiation.

Cell attachment via integrin binding to RGD sequences is known to initiate a plethora of signaling cascades, including activation of tyrosine phosphorylation of ERK/MAP kinase that regulates the cell cycle progression to proliferation [66,67]. FAs are sites where mechanical stress is converted to biochemical signals, the translation of mRNA into proteins has been shown to occur at FAs upon cell attachment and spreading via integrin binding [35,68]. Therefore, FA disassembly may inhibit recruitment of translational apparatus such as ribosomes and transcripts to the cell-substrate contacts.

The results of our study confirm that substrate-dependent preferential integrin binding negatively affected chondrocyte

differentiation. The clustering of proteoglycan receptors has also been shown to activate PKC-α, which is an intracellular marker of chondrocyte differentiation [32,40,69,70]. Lower PKC-α expression profiles demonstrated the dedifferentiation of chondrocytes on 300 g/mol substrates. Our PKC-α expression data reaffirm the maintenance of the chondrogenic phenotype on 1000 g/mol PEG polymers, which is supported by the coexpression of type II collagen and CD44.

The variation in p-FAK expression over the range of polymer substrates demonstrated the correlation of FA dynamics and turnover with integrin expression. Furthermore, since actin stress fiber assembly is also required for FAK phosphorylation [71], p-FAK is a useful parameter by which to compare the level of actin organization in chondrocytes cultured on the different polymer compositions and can thus serve as a marker for the degree of chondrocyte dedifferentiation.

RhoA is known to induce actomyosin-based contractility, resulting in the assembly of FAs and actin stress fibers that transmit tension to integrins via mediating molecules. RhoA is recruited to FAs by FAK [35,49,72]. Further downstream, RhoA itself has been shown to be involved in the recruitment of vinculin to focal adhesions [30]. Our expression data for vinculin and FAK correlated with Rho, but conversely with type II collagen and PKC-α, suggesting that the composition of polymer biomaterials does affect the differential expression of signaling proteins in chondrocytes. Furthermore, the use of molecules such as TN could be a novel method to modify cell shape by outside–in signaling without the application of external mechanical cues or toxic chemicals that are internalized in order to induce phenotypic changes that may be required for tissue regeneration. In the future, a combination of these strategies could be employed to reestablish the differentiated phenotype of expanded chondrocytes before, or concomitant with, seeding within polymer scaffolds for tissue engineering.

Conclusions

Chondrocyte phenotype can be modified by its extracellular environment via differential receptor binding and cytoskeleton organization. Analysis of matrix and focal adhesion components of chondrocytes grown on polymers with different chemistries demonstrated that cell adhesion and its subsequent signaling cascades are responsible for maintenance or loss of the chondrogenic phenotype, an understanding of which is a requisite for engineering substrate-induced cellular response.

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