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The differential proliferative response of fetal and adult human skin fibroblasts to TGF-β is retained when cultured in the presence of fibronectin or collagen ☆

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ABSTRACT

Background: Transforming growth factor-β is a multifunctional and pleiotropic factor with decisive role in tissue repair. In this context, we have shown previously that TGF-β inhibits the proliferation of fetal human skin fibroblasts but stimulates that of adult ones. Given the dynamic reciprocity between fibroblasts, growth factors and extracellular matrix (ECM) in tissue homeostasis, the present study aims to investigate the role of fibronectin and collagen in the proliferative effects of TGF-β on fetal and adult cells.
Methods: Human fetal and adult skin fibroblasts were grown either on plastic surfaces or on surfaces coated with fibronectin or collagen type-I, as well as, on top or within three-dimensional matrices of polymerized collagen. Their proliferative response to TGF-β was studied using tritiated thymidine incorporation, while the signaling pathways involved were investigated by Western analysis and using specific kinase inhibitors.
Results: Fetal skin fibroblast-proliferation was inhibited by TGF-β, while that of adult cells was stimulated by this factor, irrespective of the presence of fibronectin or collagen. Both inhibitory and stimulatory activities of TGF-β on the proliferation of fetal and adult fibroblasts, respectively, were abrogated when the Smad pathway was blocked. Moreover, inhibition of fetal fibroblasts was mediated by PKA activation, while stimulation of adult ones was effected through the autocrine activation of FGF receptor and the MEK–ERK pathway.
Conclusions: Fetal and adult human skin fibroblasts retain their differential proliferative response to TGF-β when cultured in the presence of fibronectin and unpolymerized or polymerized collagen.
General significance: The interplay between TGF-β and ECM supports the pleiotropic nature of this growth factor, in concordance with the different repair strategies between fetuses and adults. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

Wound healing is a homeostatic mechanism aiming at the restoration of the tissue's structural and functional integrity after injury. Immediately after wound formation, platelets and other immunocytes release a plethora of growth factors that regulate the various stages of the repair process [1,2]. A prominent factor among them is transforming growth factor-β (TGF-β), a member of a superfamily of conserved polypeptides with multiple biological functions [3]. TGF-β is essential for proper development and the maintenance of tissue homeostasis and function due to its ability to regulate a variety of cellular responses, including cell proliferation, migration, differentiation, apoptosis, immunomodulation, extracellular matrix (ECM) accumulation/formation and tissue repair [4–6]. On the other hand, there is increasing evidence that the subversion of TGF-β action can contribute to developmental defects and to a vast array of diseases, such as fibrosis or cancer [5,7–9]. Concerning wound healing, TGF-β affects nearly all aspects of tissue repair, such as cell proliferation, chemotaxis, production, accumulation and remodeling of ECM components, fibroblast-to-myofibroblast differentiation or angiogenesis [10].

TGF-β performs its biological actions via two serine/threonine kinase transmembrane receptors, namely the TGF-β receptor type II (TβR-II) and type I (TβR-I) that act in sequence. TβR-I activates several intracellular signaling pathways, most important being the Smad pathway. In particular, activated TβR-I serves as a docking site for receptor-associated Smad2 and Smad3 which, when phosphorylated, form a complex with the common Smad4 protein, translocate to the nucleus

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and stimulate the transcription of several target genes [11,12]. TGF-β also activates several non-Smad signaling pathways, including members of the mitogen-activated protein kinase (MAPK) family, protein kinases A and C (PKA and PKC), or phosphatidylinositol-3 kinase (PI-3K) [13–19].

TGF-β is the prototype of a pleiotropic factor, as it can exert numerous biological functions. Furthermore, its action is clearly dependent on the target cell type and the specific culture conditions [2,20,21]. Especially concerning cell proliferation, TGF-β is a potent inhibitor of several cell types, like epithelial and endothelial of hematopoietic cells, while it has been found to be stimulatory for mesenchymal cells, such as fibroblasts [9]. Its inhibitory effect has been ascribed to the upregulation of cyclin-dependent kinase inhibitors, such as $p21^{WAF1}$, $p15^{INK4B}$ and $p57^{Kip2}$ [11], while its stimulatory effect has been linked to the down-regulation of $p21^{WAF1}$ or the secretion and the subsequent autocrine action of other growth factors [22–27].

It is well known that profound differences exist between fetal and adult wound healing in mammals. While in adults healing is characterized by intense inflammation, collagenous scar formation and contraction, fetal healing is marked by a minimal inflammatory reaction and the absence of scar formation in the first two trimesters of gestation [28]. These differences can be attributed not only to the unique fetal environment, but also to the intrinsic differences of the tissue in these two developmental stages [29]. In this vein, we have shown that fetal and adult human skin fibroblasts respond differently to TGF-β; while TGF-β inhibits fetal cells, it stimulates the proliferation of fibroblasts from adult donors [30]. Furthermore, we have investigated the mechanisms underlying these differential effects and have shown that TGF-β inhibits fetal cells by the activation of PKA and the subsequent up-regulation of $p21^{WAF1}$ and $p15^{INK4B}$. On the other hand, the same factor stimulates adult skin fibroblasts by the release of fibroblast growth factor-2 (FGF-2) and, via its receptor FGFR-1, the consequent activation of the MEK–ERK pathway [31].

Notably, all the abovementioned studies have been performed under conventional culture conditions, i.e. with cells growing on plastic surfaces. These conditions are far from those that the cells experience in vivo, where they are surrounded by ECM components. In addition, this extracellular environment is not stable. For example, during the early phases of tissue repair the fibroblasts grow and migrate on a provisional matrix containing mainly fibronectin, while at the late stages of the repair process or in the intact tissue they are embedded within a matrix containing mostly fibrils of polymerized collagen [1]. Fibroblasts are the main producers of ECM in the connective tissue. Additionally, there is a dynamic reciprocity between fibroblasts and ECM, as the latter clearly affects the function of the former [32,33], and fibroblast–matrix interactions are crucial for proper healing and for the maintenance of tissue homeostasis [34].

Accordingly, our aim was to investigate the role of ECM components in the differential effect of TGF-β on the proliferation of fetal and adult human skin fibroblasts. To this end, we compared the action of TGF-β on cells grown either on plastic surfaces or on surfaces coated with ECM components, i.e. fibronectin or collagen. Furthermore, we plated the cells on top or within three-dimensional matrices of polymerized collagen, the latter simulating better their normal environment in vivo [35]. The molecular mechanisms activated by TGF-β in cells cultured on plastic or in the presence of ECM components have also been compared.

2. Materials and methods

2.1. Cells and cell culture conditions

In the present study two commercially available normal human skin fibroblast strains have been used: one from an 18-week fetus (Detroit 551, ATCC, Rockville, USA) and a neonatal foreskin strain (AG01523c, Coriell Institute for Medical Research, Camden, NJ, USA) [30,31]. Furthermore, primary cultures of human fetal and adult skin fibroblasts were developed in our laboratory after tissue dissection, overnight incubation at 37 °C with 1 mg/ml crude type I collagenase (Biochrom, Berlin, Germany) and centrifugation [36,37]. Prior to biopsy, patients gave their written informed consent, while the procedures were under the approval of the ethical committee of the NCSR "Demokritos". All cell strains were cultured in DMEM supplemented with 10% FBS (cell culture media were from Biochrom) in an environment of 5% CO_2, 85% humidity, and 37 °C, and subcultured once a week at a 1:2 split ratio, using a trypsin–citrate solution (0.25%–0.3%, respectively). Cell counting after trypsinization was performed using a Coulter counter. Cells were tested periodically and found to be mycoplasma-free.

2.2. Cell culture in the presence of ECM constituents

Fibronectin from bovine plasma (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was purchased as a sterile solution (1 mg/ml) in Tris–HCl 50 mM (pH: 7.50), NaCl 500 mM buffer. In order to coat the culture dishes with fibronectin, this solution was further diluted in the above buffer to a final concentration of 1.33 μg/ml and a volume enough to cover completely the bottom of the dishes was incubated in the CO_2-incubator for 72 h. The fibronectin solution was aspirated and a wash with serum-free culture medium was performed before the plating of the cells.

Collagen was extracted from rat-tail tendons according to a modification of the method of Bell et al. [38]. Briefly, tendons were solubilized under aseptic conditions in 0.1% (v/v) acetic acid, for 48 h, at 4 °C. The solution was centrifuged at 10,000 rpm in a Sorvall (DuPont) model RC-5C centrifuge in an SS-34 rotor for 90 min and the supernatant was stored at 4 °C. This stock solution contained approx. 4 mg/ml total protein, and consisted primarily of collagen type I. Alternatively, a commercially available rat-tail collagen type I solution was used (BD Biosciences, Bedford, MA, USA) containing 4.01 mg/ml total protein in 0.02 N acetic acid. Equivalent results were obtained using both collagen type I solutions.

An unpolymerized collagen coating on the culture dishes was obtained after a three-hour incubation in room temperature with collagen type I diluted to a final concentration of 50 μg/ml, followed by a wash with serum-free culture medium before the plating of the cells, as described above for fibronectin. A thin collagen-gel coating of the dishes was produced by premixing the collagen solution with DMEM 10× and $NaHCO_3$ 7.5% at a ratio of 17:2:1, layering it on the culture dishes and leaving it for 30 min at 37 °C for polymerization, before plating the cells directly on the gel. In order to form three-dimensional fibroblast-populated collagen gels, in the above premix of collagen solution–DMEM 10×–$NaHCO_3$ 7.5% (17:2:1) a pellet of fibroblasts (3.5×10^5 cells/ml of solution) and FBS (at a final concentration of 0.1%) were added, mixed gently and layered on the culture dishes. After 30 min at 37 °C for polymerization, culture medium containing 0.1% FBS was layered on top of the gels.

2.3. DNA synthesis assay

Regarding experiments on plastic or fibronectin- or collagen-coated surfaces, cells were plated at a density of 2×10^4 cells/cm^2, in DMEM containing 10% FBS, allowed to grow until confluence, and then synchronized in DMEM containing 0.1% FBS. After two days of serum deprivation, fresh medium was added to the quiescent cultures, along with 5 ng/ml human recombinant (h.r.) transforming growth factor-β1 (TGF-β1; Biochrom), 10 ng/ml h.r. platelet-derived growth factor-BB (PDGF-BB; R&D Systems Europe, Abingdon, UK) or a mixture of both; [methyl-^{3}H]thymidine (Moravek Biochemicals Inc., CA, USA) at 0.15 μCi/ml, 25 Ci/mmol, was also included in the medium. In certain experiments h.r. basic fibroblast growth factor (bFGF; R&D Systems Europe) at a final concentration of 10 ng/ml was also used. After 48 h of incubation, the culture medium was decanted; the cells were washed

with phosphate buffered saline (PBS), fixed with 10% ice-cold trichloroacetic acid, washed extensively under running tap water and air-dried. DNA was solubilized by the addition of 0.3 N NaOH/1% SDS and the lysates were subjected to scintillation counting [39].

For experiments with three-dimensional fibroblast-populated collagen gels (see above) which have been left in quiescence for 48 h in DMEM 0.1% FBS, growth factors and [methyl-^{3}H]thymidine were added as already described and left for a further 48-h incubation. Then, the medium was aspirated and the collagen gels were digested for 1 h at 37 °C, with a crude collagenase solution (1 mg/ml in 130 mM NaCl, 10 mM $CaCl_2$, 10 mM HEPES, pH 7.2). The cells were collected by centrifugation (1000 ×*g*, 5 min) and lysed with 0.3 N NaOH/1% SDS solution. Ice-cold TCA (f.c. 10%) was added to the lysates, which were then filtered through glass-fiber filters (Sigma). The filters were air-dried and subjected to scintillation counting, as previously described [40].

2.4. Western analysis

Human fetal or adult skin fibroblasts were synchronized in two- or three-dimensional cultures, as described above for the DNA synthesis assay, and treated with TGF-β for the indicated time-periods. For the collection of cell-lysates from two-dimensional cultures, these were washed with ice-cold Tris buffered saline (TBS: 10 mM Tris–HCl, pH 7.4, 150 mM NaCl) and scraped immediately in hot SDS-PAGE sample buffer, i.e. 62.5 mM Tris, pH 6.8, 6% w/v SDS, 2% v/v glycerol, 5% v/v 2-mercaptoethanol, 0.0125% w/v bromophenol blue, protease- and phosphatase-inhibitor cocktails (Sigma). Following sonication for 15 s, the samples were clarified by centrifugation and stored at −80 °C until use.

For the collection of cell-lysates from three-dimensional cultures, gels were washed with ice-cold TBS, carefully detached from the culture dishes and transferred to Eppendorf tubes. After a brief centrifugation (10,000 ×*g*, 3 min, 4 °C), the supernatant was discarded, gels including the cells were compacted to pellets, hot SDS-PAGE sample buffer was added to the pellets and the same procedure as in the case of two-dimensional culture-lysates was followed (see above).

The lysates were separated on SDS-PAGE (9% and 10% for Smad and TβR analyses, respectively) and the proteins were transferred to PVDF membranes (Amersham). The membranes were blocked with 5% (w/v) non-fat dried milk in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20 (TTBS) buffer and incubated with the appropriate primary antibodies, i.e. rabbit anti-TβR-I (V-22; Santa Cruz Biotechnology, Inc., Heidelberg, Germany), rabbit anti-TβR-II (L-21; Santa Cruz Biotechnology), rabbit anti-phospho-Smad2 (Ser465/467) and anti-Smad2 antibodies (both generously provided by Dr. A. Moustakas), rabbit anti-phospho-Smad3 (Ser423/425) and anti-Smad3 antibodies (both from Cell Signaling Technology, Hertfordshire, UK), mouse anti-Smad4 (B-8; Santa Cruz Biotechnology), or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (6C5; Santa Cruz Biotechnology). After washing with TTBS, the membranes were incubated with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated goat secondary antibody (Sigma) and washed again with TTBS and the immunoreactive bands were visualized by chemiluminescence (ECL Kit, Amersham Biosciences) according to the manufacturer's instructions on a Fujifilm LAS-4000 luminescent image analyzer (Fujifilm Manufacturing USA Inc., Greenwood, SC, USA).

2.5. Treatment with specific inhibitors

The inhibitors SU-5402 and H-89 (both from Calbiochem-Merck KGaA, Darmstadt, Germany), and PD98059 (Sigma) were applied to the serum-deprived cultures and after 45 min growth factors were also added. For assessing the effect of kinase inhibitors on DNA synthesis, [methyl-^{3}H]thymidine was included in the incubation medium (see above) for further 48 h, and DNA synthesis was estimated. The final concentrations of the inhibitors used in the present study were: 4 μM H-89, 25 μM PD98059, and 20 μM SU-5402. These were selected based on the literature and on previous studies in our laboratory.

2.6. Smad4 knocking-down mediated by small interfering RNA (siRNA)

Silencing of the Smad4 gene was achieved through the use of a siRNA (siSmad4: 5′-GGUCUUUGAUUUGCGUCAGtt-3′) designed to specifically target and degrade the gene's transcribed mRNA sequence [41]. The siSmad4, as well as the pre-designed scramble (5′-UAA UGU AUU GGA ACG CAU A-3′), was supplied by Eurofins MWG Operon (Ebersberg, Germany). Transfection of human fibroblasts was performed as described before [42,43] with 50 nM of either scrambled or siSmad4 sequence using Lipofectamine 2000 (Invitrogen, Paisley, UK) following the manufacturer's instructions. Five hours after transfection, the medium was aspirated and replaced by DMEM supplemented with 10% (v/v) FBS for 48 h before the collection of protein extracts that were subjected to Western analysis for Smad4. For DNA synthesis experiments, transfected cells were serum deprived for 24 h and TGF-β was added along with [methyl-^{3}H]thymidine for another 48 h.

3. Results

3.1. The response of human fetal and adult skin fibroblasts to TGF-β when cultured in the presence of fibronectin or collagen

We have already shown that TGF-β regulates in a differential manner the proliferation of fetal and adult human skin fibroblasts plated on conventional plastic surfaces [30,31]. As these conditions are far from those occurring in vivo, the aim of this work was to investigate the role of ECM components especially those prevailing in the various phases of wound repair. Accordingly, we initially studied the role of fibronectin, which represents the main component of the provisional matrix, the first ECM to be deposited in the wound space [1]. As we have already reported, TGF-β inhibits the DNA synthesis in fetal fibroblasts cultured on plastic and also inhibits the stimulatory effect of PDGF, another growth factor released by platelets and other immunocytes after wound formation. On the other hand, TGF-β stimulates the DNA synthesis of adult fibroblasts and has a synergistic effect with PDGF when the two factors are added simultaneously. Interestingly, this differential response of fetal and adult fibroblasts to TGF-β remained unaltered when cells were cultured on fibronectin-coated dishes (Fig. 1). Furthermore, the intensity (inhibitory or stimulatory, respectively) seemed to be similar when the cells were cultured on both substrata.

Next, we assessed the role of collagen, the major ECM component of the granulation tissue and of the intact dermis [1]. As can be seen in Fig. 2, the inhibitory and stimulatory effects of TGF-β on fetal and on adult fibroblasts, respectively, remained when cells were cultured on dishes covered with collagen. We then investigated the role of polymerized collagen gels on the effect of TGF-β. First we plated the cells in 2-D cultures on top of collagen gels. Under these conditions, TGF-β inhibited fetal and stimulated adult fibroblasts. Finally, we tested the effect of TGF-β on cells grown in three dimensional collagenous matrices, the latter mimicking the conditions of granulation tissue [44]. Once again, in these gels TGF-β inhibited fetal cells, while it stimulated adult fibroblasts (Fig. 2). PDGF always exerted a proliferative effect in both cell types under all experimental conditions. The action of PDGF was inhibited by TGF-β in fetal cells. On the contrary, the simultaneous addition of the two factors in adult cells led to a synergistic effect (Fig. 2).

3.2. Expression of TGF-β receptors and activation of Smad pathways in fetal and adult fibroblasts cultured in the presence of polymerized collagen

Subsequently, we attempted to identify the molecular mechanisms underlying the observed TGF-β-mediated responses of fetal and adult cells. Given that cells were shown to respond similarly to the growth

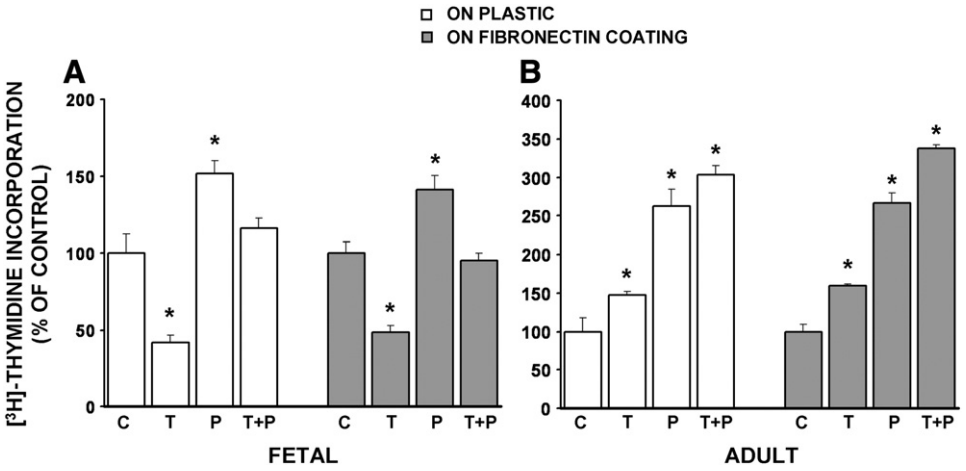

Fig. 1. TGF-β retains its antiproliferative and mitogenic effects on human fetal and adult skin fibroblasts, respectively in the presence of fibronectin. Fetal (A) and adult (B) cells were plated on plastic- and fibronectin-coated surfaces until confluence and synchronized in DMEM containing 0.1% FBS for 48 h before the addition of TGF-β1 and PDGF-BB along with [methyl-^{3}H]thymidine for another 48 h. [^{3}H]thymidine incorporation was estimated by scintillation counting as described in Materials and methods. Mean values (±standard deviation) from three independent experiments performed in triplicates are depicted. Asterisks represent statistically significant differences in comparison to the respective control (t test, $p < 0.05$).

factor in all culture systems used above, experiments investigating the biochemical pathways implicated in these responses were selectively conducted from this point on in three-dimensional collagen gels that better reflect the in vivo conditions of the granulation tissue.

We first studied the expression of TGF-β receptors in fetal and adult fibroblasts cultured on plastic or within collagen gels. Lysates were derived from the same number of cells in every condition to allow comparison (Fig. 3). Using an antibody recognizing the cytoplasmic domain of

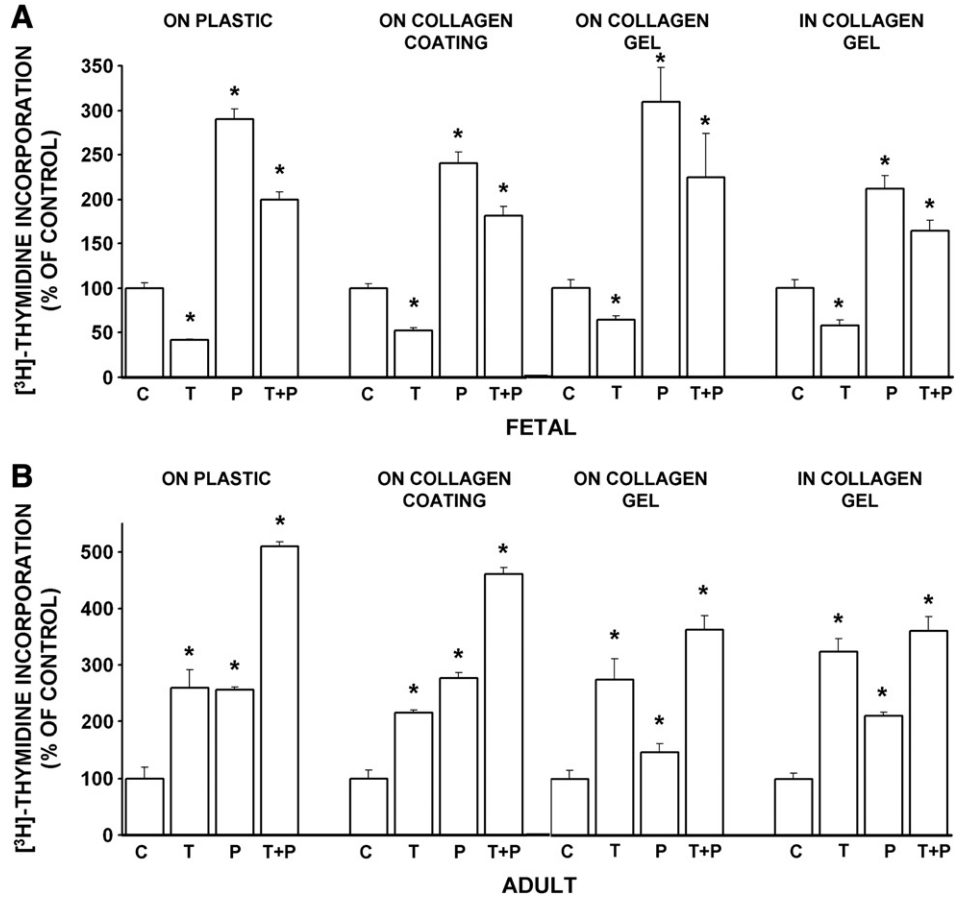

Fig. 2. Effect of TGF-β on the proliferative potential of human fetal and adult skin fibroblasts in the presence of collagen. Fetal (A) and adult (B) cells were plated on plastic and on collagen-coated surfaces, as well as, on and in collagen gels. Cells were incubated in DMEM containing 0.1% FBS for 48 h and growth factors were added along with [methyl-^{3}H]thymidine for another 48 h. DNA synthesis was assessed as described in Materials and methods. Mean values (±standard deviation) from three independent experiments performed in triplicates are presented. The asterisks show statistically significant differences of samples compared to the respective controls (t test, $p < 0.05$).

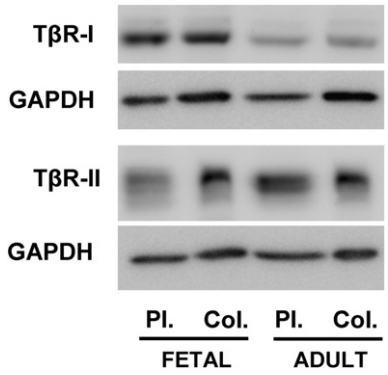

Fig. 3. Estimation of TGF-β receptors' basal expression levels in human fetal and adult skin fibroblasts cultured in three-dimensional collagen gels. Fetal and adult cells were plated on plastic and in collagen gels and then synchronized in DMEM containing 0.1% FBS for 48 h. Cell lysates from two-dimensional cultures were collected in SDS-PAGE sample buffer by scraping after a wash with ice-cold Tris buffered saline (TBS). Cells from three-dimensional cultures were recovered by centrifugation from the detached gels after a washing step with ice-cold TBS in the same SDS-PAGE sample buffer. Lysates were subjected to Western blot analysis for TGF-β receptor I (TβR-I) and II (TβR-II), while blots for GAPDH served as loading controls. Representative blots from two independent experiments are depicted here.

TβR-II, we showed that adult fibroblasts express higher levels of this receptor than fetal cells on plastic. On the other hand, the expression of TβR-II was similar in fetal and adult cells within collagen gels. Regarding TβR-I we used an antibody recognizing its cytoplasmic domain and demonstrated that its expression is lower in adult cells cultured on plastic. These differences in TβR-II basal expression levels remained when the two cell types were cultured in gels of polymerized collagen.

As we have already reported [30,31], Smad2 and Smad3 are immediately phosphorylated in both fetal and adult fibroblasts cultured on plastic surfaces after stimulation with TGF-β, with a peak at 30 min to 1 h after growth factor addition (see also Fig. 3). Here we showed that both Smad2 and Smad3 are phosphorylated in fetal and adult cells 1 h after TGF-β addition (Fig. 4). Furthermore, the kinetics of phosphorylation/dephosphorylation remained similar in both culture conditions (not shown here).

3.3. Molecular mechanisms of the differential proliferative response of fetal and adult fibroblasts to TGF-β when cultured in collagen gels

Since Smad pathways are considered central mediators of TGF-β-signals to the nucleus [19], we have studied here the contribution of

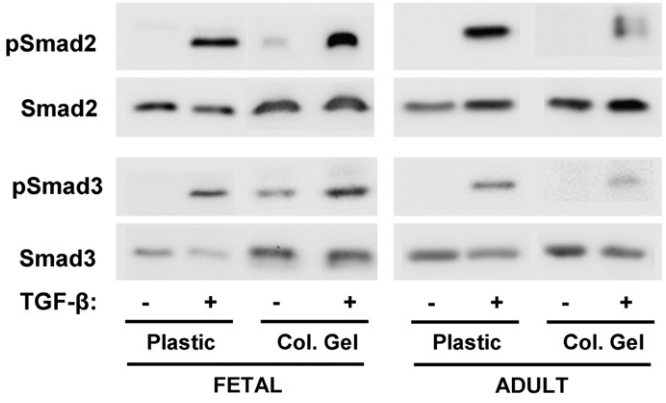

Fig. 4. Estimation of the TGF-β-induced phosphorylation levels of Smad2 and Smad3 in human fetal and adult skin fibroblasts cultured in three-dimensional collagen gels. Fetal and adult cells were seeded on plastic and in collagen gels before synchronization with DMEM containing 0.1% FBS for 48 h. Cell lysates from two- and three-dimensional cultures were subjected to Western blot analysis for the phosphorylated Smad2 and Smad3. Western blots against the non-phosphorylated forms of Smads were performed to verify equal loading. Two independent experiments were conducted and representative blots are shown.

the common mediator Smad4 to the proliferative effects of TGF-β on fetal and adult fibroblasts. Incubation with the siRNA sequence that specifically targets the Smad4 mRNA results in >90% reduction of the basal Smad4 protein levels in both fetal and adult cells (Fig. 5A). This loss of Smad4 expression led to the abrogation of the inhibitory and stimulatory effects of TGF-β on fetal and adult fibroblasts, respectively (Fig. 5B).

On the other hand, we have shown previously that the inhibitory action of TGF-β on fetal fibroblasts cultured on plastic surfaces is linked to the activation of PKA, while the TGF-β-induced proliferation of adult fibroblasts is mediated by the release of FGF-2 and the consequent activation of the MEK–ERK pathway via FGFR-1 [31]. Accordingly, we tested whether the same molecular mechanisms were activated when human fibroblasts were cultured in gels of polymerized collagen. As shown in Fig. 6, the specific PKA inhibitor H-89 blocked the inhibitory effect of TGF-β on fetal fibroblasts cultured in collagen gels, confirming the contribution of PKA under these culture conditions, as well.

Furthermore, as shown in Fig. 7, FGF-2 intensely stimulated DNA synthesis in human adult fibroblasts cultured in collagen gels. Moreover, SU-5402, a specific inhibitor of FGFR-1, was able to completely annul the action of both FGF-2 and TGF-β (Fig. 7), indicating that the action of TGF-β is mediated by the action of FGF-2 when the cells are cultured in collagen gels, in keeping with our previous observations with cells cultured on plastic surfaces [31]. Finally, PD98059, the specific inhibitor of the MEK–ERK pathway, completely abolished the stimulatory effect of TGF-β on DNA synthesis, as well (Fig. 7).

4. Discussion

Mammalian fetuses and adults follow different strategies for their wound repair. While in the adults the healing of the injured tissue is characterized by an acute inflammation, collagen deposition and remodeling and eventually scar formation, in the fetuses tissue repair is marked by a minimal inflammatory response and the absence of contraction and scar formation [45]. Several studies, mainly by utilizing grafts of fetal or adult skin, have provided evidence indicating that these differences are not primarily due to the unique fetal environment, but to the differentiation of the fetal tissue [29,46–48]. Thus, several studies have shown that fetal skin fibroblasts display major differences, e.g. concerning migration, contraction, and secretion of ECM components, from adult fibroblasts, when cultured under identical conditions in vitro [10,49–53]. In accordance, skin fibroblasts from these two developmental stages respond differently to TGF-β, a major growth factor in the healing response. This factor stimulates the proliferation of adult fibroblasts, while it inhibits fetal cells by using completely different mechanisms [30,31].

A large number of studies have indicated a dynamic interaction between two major components of the healing process, i.e. the ECM and growth factors [33]. Growth factors, and especially TGF-β, regulate the synthesis, deposition, degradation and remodeling of ECM [54–58]. On the other hand, ECM can regulate the function of growth factors by their sequestration, storage and protection [59]. However, although this "dynamic reciprocity" [32] is well accepted, the majority of the in vitro studies concerning the effect of growth factors has been performed on conventional plastic surfaces, a condition that leads to an "activated" state of most cell types.

TGF-β affects nearly all phases of wound healing. It is released from the α particles of platelets during the formation of the wound [2], as well as by immunocytes and resident cells (e.g. fibroblasts) in all the duration of the repair process, and regulates several functions from chemotaxis and cell proliferation to contraction and ECM deposition and remodeling [60]. On the other hand, ECM components that are synthesized by TGF-β, like decorin or biglycan, can bind and inactivate TGF-β, thus forming a negative feedback loop [33,61,62].

In this study we investigated the role of TGF-β on the differential proliferative effect it exerts on fetal and adult skin fibroblasts, when these cells are cultured in the presence of two major components of

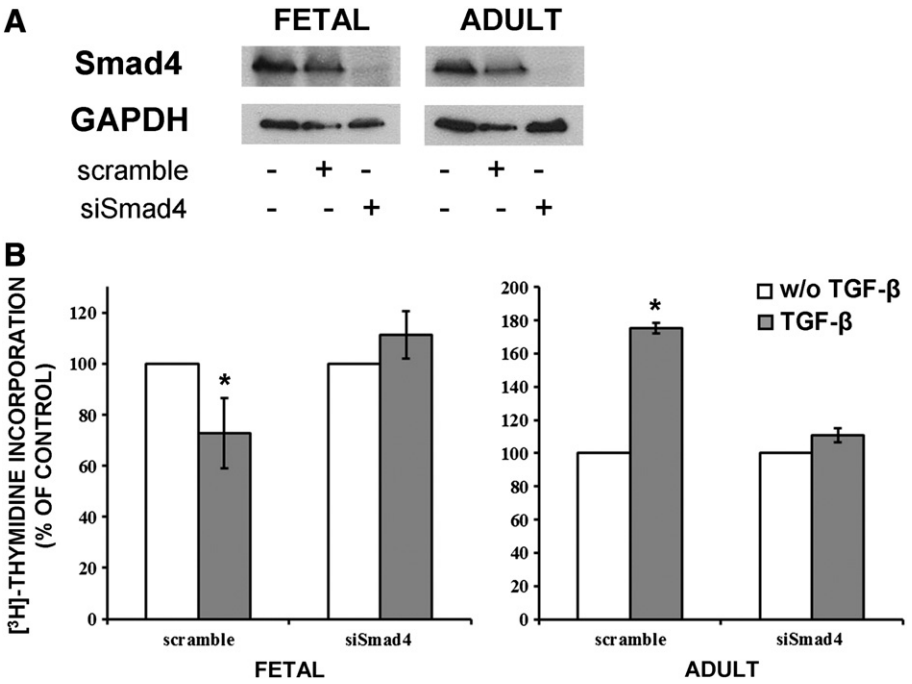

Fig. 5. Inhibition of Smad4 expression abrogates the TGF-β-induced antiproliferative and mitogenic effects on fetal and adult human fibroblasts, respectively. Fetal and adult cells were transfected with 50 nM scramble or siSmad4; in (A) the Smad4 protein levels were detected by Western analysis after incubation for 48 h, while GAPDH was used as loading control; in (B) DNA synthesis was estimated as described in Materials and methods. Mean values (±standard deviation) from three independent experiments performed in triplicates are depicted. Asterisks represent statistically significant differences in comparison to the respective control (t test, $p < 0.05$).

ECM, i.e. fibronectin or collagen. Fibronectin is an adhesive glycoprotein that is deposited in the initial phase of wound healing from blood plasma, while it can be also synthesized locally by resident cells [63,64], and it represents a major component of the provisional matrix. Here we showed that fetal and adult skin fibroblasts cultured on fibronectin-coated dishes have the same response to TGF-β as when they are cultured on conventional plastic dishes. Next, we tested the role of collagen, i.e. the major ECM component in the granulation tissue, in the response of cells to TGF-β. Once again, fetal and adult fibroblasts plated on collagen-coated dishes maintained their response (inhibitory and stimulatory, respectively) to TGF-β. We repeated the experiments using gels of polymerized collagen, as polymerization may reveal "cryptic" domains that could affect cell function. In this direction, it has been previously reported that monomer collagen supports the proliferation

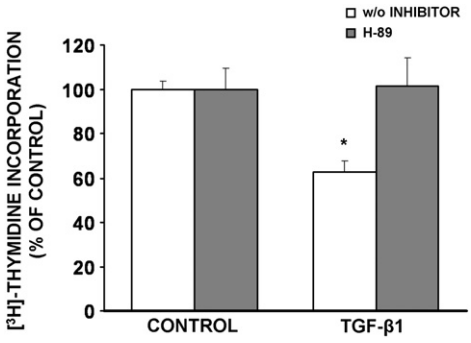

Fig. 6. The inhibitory effect of TGF-β on human fetal skin fibroblasts cultured in collagen gels is mediated by PKA. Fetal skin fibroblasts were plated in collagen gels and then incubated in DMEM containing 0.1% FBS for 48 h. The specific PKA inhibitor H-89 was applied to the serum-deprived cultures for 45 min before the addition of TGF-β1 along with [methyl-^{3}H]thymidine followed by a further incubation for 48 h, and DNA synthesis was assessed as described in Materials and methods. Results presented here are the means (±standard deviation) from three independent experiments performed in triplicates, while statistically significant differences in comparison to the respective control (t test, $p < 0.05$) are shown by asterisks.

of arterial smooth muscle cells, while on polymerized type I collagen fibrils the cells remain arrested in the G1 phase of the cell cycle [65]. When cells (fetal and adult) were cultured on top of the gels or within the gels of polymerized collagen – a system that better simulates the conditions prevailing in vivo [35] – their response to TGF-β remained unaltered. Interestingly, in all cases PDGF exerted a stimulatory effect in both fetal and adult fibroblasts. Previous studies on human dermal and gingival fibroblasts cultured in collagen gels are in agreement with our findings regarding the ability of PDGF to stimulate DNA synthesis [66,67]. However, in the same reports it is mentioned that TGF-β is unable to stimulate proliferation. Interestingly, while polymerized collagen can drastically reduce collagen production after stimulation with TGF-β [40], it does not affect their proliferative response to this growth factor, indicating that the feedback loop inhibiting collagen synthesis is mediated via other signaling pathways, probably regulated by collagen-binding integrins.

We further studied the mechanisms underlying the effect of TGF-β in cells cultured within collagen gels. Our data showed that under these conditions both fetal and adult cells express TGF-β receptors I and II and are able to activate Smad2 and Smad3 by phosphorylation. Concerning the inhibitory effect of TGF-β on fetal cells, we demonstrated that in cells grown on plastic, this is due to the activation of PKA [31]. A specific PKA inhibitor (H-89) was able to annul this inhibitory effect in collagen gels, as well. On the other hand, we have reported that the stimulatory effect of TGF-β on adult fibroblasts cultured on plastic is due to a release of FGF-2 and the subsequent activation of the MEK–ERK pathway, via the FGFR-1 [31]. In this study we showed that FGF-2 is able to stimulate adult skin fibroblasts in collagen gels in contrast to previous results [67]. Furthermore, the specific inhibitors against FGFR-1 and the MEK–ERK pathway were able to block the stimulatory effect of TGF-β. Interestingly, both inhibitory and stimulatory activities of TGF-β on the proliferation of fetal and adult fibroblasts, respectively, were abrogated when Smad4 expression was blocked, suggesting that the Smad pathway is also required for both PKA activation in fetal cells and FGF-2 release in adult ones. Collectively, the aforementioned findings suggest that TGF-β regulates proliferation of fetal and adult

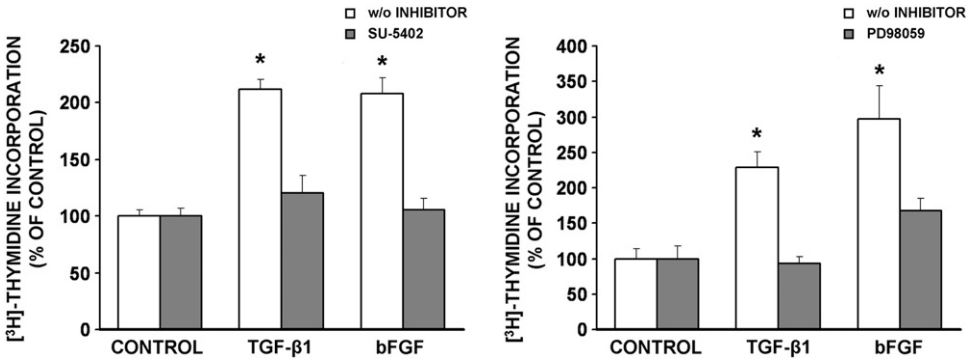

Fig. 7. The blockade of the FGF-2 receptor 1 and of the MEK/ERK pathway abolishes the stimulatory action of TGF-β on human adult skin fibroblasts cultured in collagen gels. Adult skin fibroblasts were plated in collagen gels and incubated with DMEM containing 0.1% FBS for 48 h. SU-5402 and PD98059 were added to the cultures for 45 min before the addition of TGF-β1 or FGF-2 along with [methyl-^{3}H]thymidine and, after a further 48-h-incubation, DNA synthesis was assessed as described in Materials and methods. Mean values (±standard deviation) from three independent experiments performed in triplicates are depicted. Asterisks represent statistically significant differences in comparison to the respective control (t test, $p < 0.05$).

fibroblasts by a similar mechanism when cells are cultured on plastic surfaces or within gels of polymerized collagen.

In conclusion, in this work we provide evidence that fetal and adult skin fibroblasts retain their differential response to TGF-β when cultured in the presence of fibronectin and unpolymerized or polymerized collagen. This finding indicates that TGF-β is able to stimulate the proliferation of adult skin fibroblasts being on the provisional matrix or in the granulation tissue. Similarly, in the fetus, where fibronectin and collagen are integral parts of their ECM during wound healing, it seems that TGF-β can regulate the proliferation of fibroblasts, most probably towards a controlled tissue repair. Even though the wound matrix is very complex and it is difficult to develop culture systems that fully reflect the actual conditions prevailing in the tissue, the systems used in this study are much closer to the in vivo environment compared to the conventional plastic surfaces used in previous reports so far. Future upgrading of these in vitro approaches using combinations of several ECM components or even expansion of appropriate animal models could ultimately lead to a more complete understanding of the ECM–growth factor interactions in the various phases of tissue repair in fetal and adult skin.

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