FEBS Letters 430 (1998) 358–362 FEBS 20459

Mechanical stress induces DNA synthesis in PDL fibroblasts by a mechanism unrelated to autocrine growth factor action

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Received 27 May 1998

Abstract Periodontal regeneration is thought to require the proliferation of stress-sensitive periodontal ligament (PDL) fibroblast cells. The influence of physiological amounts of mechanical stretching on the DNA synthesis potential of human PDL fibroblasts was examined by means of an established, simple in vitro system of stretch application. A significant increase in the relative levels of incorporation of tritiated thymidine was observed in cultures stretched for 1–6 h. Neutralising antibodies for platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) did not blunt the DNA synthesis induction. This mitogenic response to stretch appears to be independent of an autocrine mechanism involving growth factors in general, because stretch-conditioned medium, when transferred to non-stretched fibroblasts, did not mimic the mitogenic effect of stretch.

Key words: Periodontal ligament fibroblast; Mechanical stretch; Mechanotransduction; DNA synthesis; Growth factor; Autocrine mechanism

1. Introduction

It is well-documented that mechanical load applied to a specific area of bone tissue affects it in a way that complete remodelling of the area occurs. This remodelling process causes either bone formation in tension sites or bone resorption in compression sites [1]. Many treatment modalities in orthopedics are based on this phenomenon. Particularly, in dentofacial orthopedics, certain facial and dental anomalies are successfully treated by the application of controlled systems of mechanical forces. Although data available today provide some insights into how cells sense mechanical stimuli and convert them into biochemical responses, which ultimately will lead to genetic reprogramming, the precise molecular mechanism of mechanosignal transduction is still obscure; however, there are a few clues. A mechanosensitive receptor is assumed to have some interaction with the plasma membrane in order to sense the tension of the membrane ([2] and references therein). Accordingly, integrins, heterodimeric transmembrane receptors that couple components of the extracellular matrix to the cytoskeleton, are attractive candidates for transmitting mechanical signals intracellularly. Indeed, Wang et al. [3] have demonstrated that magnetic beads coated with an integrin ligand are capable of transferring mechanical stress to the cytoskeleton. Changes in the phosphorylation state of certain proteins have been identified when hydrostatic pressure was applied to stress-sensitive mandibular condylar chondrocytes of albino rats [4]. Mechanical stretching was also found to activate rab and rho GTPases (small GTP-binding proteins that control many intracellular processes such as proliferation, vesicular trafficking as well as cytoskeletal architecture) in fibroblasts derived from human periodontium [5]. The latter cells represent the most common cell type in periodontal connective tissue and the most important for periodontium regeneration, which is considered to require periodontal ligament (PDL) cell migration and proliferation [6]. Moreover, these particular cells bear the phenotypic characteristics of osteoblast-like cells [7].

The effect of mechanical stretching on DNA synthesis is rather inductive as it has been shown in epithelial, endothelial, bone and vascular smooth muscle cells [8–11] and involves largely unknown events. In the case of vascular smooth muscle cells, it has been reported that the induction of DNA synthesis following mechanical perturbation of these cells involves an autocrine action of growth factors [11]. Platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) are regarded to act as potent biological mediators modulating numerous activities of tissue repair [12–16]. These polypeptide factors are released by platelets and other immunocytes, as well as adjacent somatic cells, thus acting in an autocrine or paracrine manner. The fundamental functions of these factors as mitogens for a wide variety of cell types have been relatively well characterized, and their effect on tissue remodelling has already been demonstrated by in vivo administration in animals and in human experimental models [17,18]. Especially for the PDL, it has been shown that PDGF can stimulate regeneration of the periodontal attachment and it has been used as part of a treatment strategy (guided tissue regenerative therapy) in order to promote periodontal regeneration of class II furcation defects [19].

The purpose of the present study was to investigate the effect of short-term mechanical stretching on the regulation of DNA synthesis (as a prerequisite of proliferation) in fibroblasts isolated from human PDL, by culturing these cells on a flexible plastic substrate and stretching the substrate by using a previously elaborated, simple system of weight application. Furthermore, the question of whether this regulation implicates the intermediary action of secreted growth factors, in particular PDGF and TGF-β, was also addressed.

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Abbreviations: PDL, periodontal ligament; GTPase or GTP-binding protein, guanosine triphosphate-binding protein; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-beta; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PKA, protein kinase A; PKC, protein kinase C; bFGF, basic fibroblast growth factor

PII: S0014-5793(98)00695-4

2. Materials and methods

2.1. General

All culture media were purchased from Seromed. [^{3}H]Thymidine (25 Ci/mmol) was obtained from Amersham. Human PDGF-AA, PDGF-BB and TGF-β1 were from Gibco. Polyclonal antibodies against human PDGF and TGF-β were purchased from R&D Systems. Staurosporine was obtained from Sigma Chemical.

2.2. Cells and cell culture conditions

Human PDL fibroblasts were obtained from explants dissected from the roots of healthy extracted third molars, as previously described [5]. They were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air, in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal calf serum (FCS) and antibiotics (penicillin, 50 U/ml and streptomycin, 50 mg/ml). The cells were subcultured by the use of trypsin-citrate solution [20] once a week at a split ratio of 1:2. Under these conditions, PDL fibroblasts exhibited a limited in vitro lifespan of approximately 20 passages. All experiments utilized primary cultures, i.e. from the third to fifth passages. Cell cultures were tested periodically and found to be mycoplasma-free.

2.3. Application of mechanical stretching to cultured cells and DNA synthesis assay

For the stretching experiments, human PDL fibroblasts were seeded on 5-cm Petriperm culture dishes (Bachofer). The bottom surface of these dishes is a gas-permeable, hydrophilic flexible polytetrafluorethylene membrane, which can be uniformly stretched by being placed over a brass spheroidal, convex template, equilibrated to 37°C [5]. The cells were grown in DMEM containing 15% FCS until they reached ~80% confluency. The cultures were then washed with phosphate-buffered saline (PBS) and the medium was changed to DMEM supplemented with 0.1% FCS, in order to remain quiescent. 24 h later, fresh medium (DMEM with 0.1% FCS) was added, along with [^{3}H]thymidine (0.15 μCi/ml), and the dishes were subjected to mechanical stretching (calculated as a 2.5% increase in the surface area, i.e. percentage of stretch = 2.5%) for the indicated times by placing a brass weight on top of the dish cover, as described previously [5]. Following stress application the cultures were further incubated unstretched for a total period of 48 h. Control cultures were incubated unstretched for the total 48-h period. At the end of the incubation the medium was aspirated and the cultures were washed with PBS, fixed with ice-cold trichloroacetic acid (10% w/v) for 10 min, washed extensively under running tap water and air-dried. Subsequently, the cells were lysed with the addition of a 1% sodium dodecyl sulfate (SDS)/0.3 N NaOH solution and the radioactivity was determined in a liquid scintillation counter, as described [21]. To study the effect of isolated growth factors and of conditioned media on PDL fibroblasts, the cells were grown in 12-multi-well plates (Corning) at ~80% confluency. They stayed quiescent for 24 h, as described above, and then fresh DMEM supplemented with 0.1% FCS was added, along with the growth factors or the conditioned media, in the presence of [^{3}H]thymidine. After a 48-h incubation, the cells were fixed, lysed and the cell lysates were subjected to liquid scintillation counting as described above. Results were expressed as the increase of [^{3}H]thymidine incorporation over that of control.

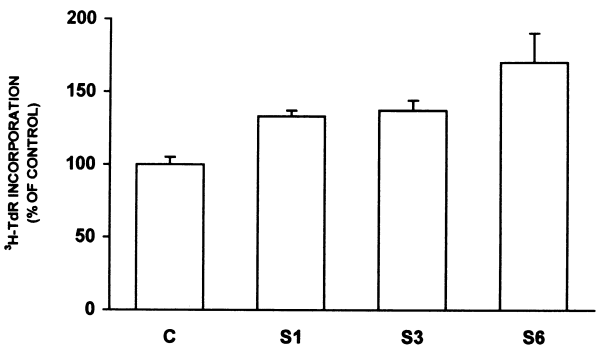

Fig. 1. Mechanical stretching induces DNA synthesis in human PDL fibroblasts. Subconfluent cultures of PDL fibroblasts remained quiescent in DMEM supplemented with 0.1% FCS. The cells were subjected to mechanical stretching for 1 h (S1), 3 h (S3) and 6 h (S6) and then left unstretched for the completion of a 48-h incubation period. Unstretched cultures were used as control (C); [^{3}H]thymidine (0.15 μCi/ml) was included in the culture medium in all cases. At the end of the incubation period [^{3}H]thymidine (^{3}H-TdR) incorporation into newly synthesized DNA was estimated, as described in Section 2. Values represent mean (±S.D.) of three individual experiments, each performed in triplicate dishes. 100% = 1898 cpm (5-cm plates).

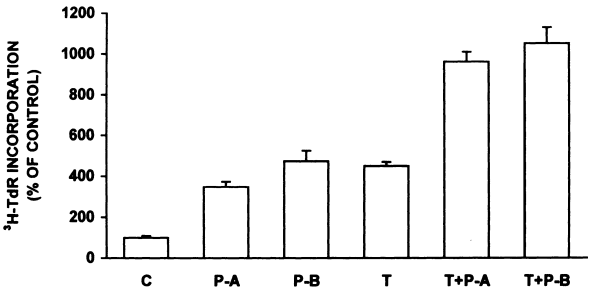

Fig. 2. Mitogenic response of human PDL fibroblasts to PDGF-AA, PDGF-BB and TGF-β1. Subconfluent cultures of PDL fibroblasts were maintained quiescent in DMEM supplemented with 0.1% FCS (C). PDGF-AA (P-A), PDGF-BB (P-B) or TGF-β1 (T) were added at a concentration of 5 ng/ml, alone or in combination (T+P-A, T+P-B). After a 48-h incubation period, in the presence of growth factors and [^{3}H]thymidine (0.15 μCi/ml), cell lysates were subjected to liquid scintillation counting as described in Section 2. Values represent mean (±S.D.) of two different experiments performed in triplicate dishes. 100% = 625 cpm (multi-well plates).

2.4. Collection of conditioned medium

Media conditioned by mechanically-stretched or control (unstretched) cultures were collected at the indicated times, centrifuged at 10 000 × *g* for 30 min at 4°C to remove all cellular debris and stored at −20°C until used. To assess their effect on PDL fibroblasts, the various conditioned media were diluted 1:1 with fresh medium (DMEM with 0.1% FCS) and their effect on DNA synthesis was determined as described above.

3. Results

Human PDL fibroblasts were subjected to mechanical stretching for periods ranging from 1–6 h, by employing the simple system of the flexible bottom culture dishes [5]. After a total incubation time of 48 h a sustained increase in novel DNA synthesis (evaluated by determining the incorporation of [^{3}H]thymidine into acid-precipitable DNA) was observed, in comparison to unstretched control cultures (Fig. 1). The induction of DNA synthesis was evident even after 1 h of stretching, while after 6 h of stretching the stimulation was more intense, i.e. 70% over the control cultures. It should be noted here that in all experiments a continuous labelling with [^{3}H]thymidine, throughout the 48-h incubation period, was used. Although the differences between control and stimulated cultures can be accentuated by using pulse labelling, the continuous labelling approach was followed inasmuch as the kinetics of this stretch-induced DNA synthesis were unknown.

It has been reported using another cell assay system that mechanical strain prompts growth of vascular smooth muscle cells via the autocrine action of growth factors, especially PDGF isoform -AA [11]. Accordingly, we investigated whether the stretch-induced DNA synthesis in PDL fibroblasts can be attributed to a secretion of autocrine growth factors, and in particular of two of the most important factors

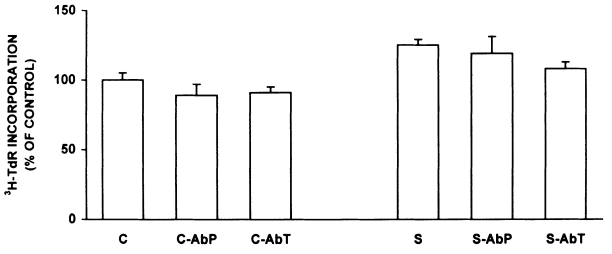

Fig. 3. Effect of anti-PDGF and anti-TGF-β antibodies on mechanical stretch-induced DNA synthesis in human PDL fibroblasts. Subconfluent cultures of PDL fibroblasts were maintained quiescent in DMEM containing 0.1% FCS (C) or were subjected to mechanical stretching for 3 h and then were left unstretched (S), in the absence or presence of polyclonal anti-human PDGF (AbP) or anti-human TGF-β1 (AbT) antibodies (10 μg/ml); [^{3}H]thymidine (0.15 μCi/ml) was included in the culture medium. After a 48-h incubation period the cells were harvested and incorporation of radioactivity into newly synthesized DNA was measured. Values represent mean (±S.D.) of two separate experiments, each performed in triplicate dishes. 100% = 1726 cpm (5-cm plates).

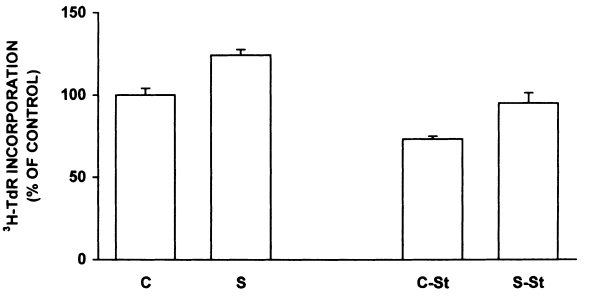

Fig. 5. Effect of staurosporine on mechanical stretch-induced DNA synthesis in human PDL fibroblasts. Subconfluent cultures of PDL fibroblasts were maintained quiescent in DMEM containing 0.1% FCS (C) or were subjected to mechanical stretching for 3 h and then were left unstretched (S), in the absence or presence of staurosporine (5×10^{-9} M) (St); [^{3}H]thymidine (0.15 μCi/ml) was also added to the culture medium. After a 48-h incubation period the cells were harvested and incorporation of radioactivity into newly synthesized DNA was assayed. Values represent mean (±S.D.) of two different experiments, each performed in triplicate dishes. 100% = 2291 cpm (5-cm plates).

in tissue homeostasis, i.e. PDGF and TGF-β. The effect of isolated PDGF and TGF-β on PDL fibroblasts is shown in Fig. 2. TGF-β1 (the most abundant isoform of TGF-β) as well as two of the PDGF isoforms (PDGF-AA and PDGF-BB) appear to be potent mitogens for PDL fibroblasts. Fig. 2 depicts the results obtained by using growth factor concentrations that are capable of inducing maximum DNA synthesis, i.e. 5 ng/ml. The effect of TGF-β1 was comparable to that of PDGF-BB while PDGF-AA elicited a less potent mitogenic response. Interestingly, the concerted action of TGF-β1 with either PDGF isoform was found to be synergistic (Fig. 2).

We then went on and subjected PDL fibroblast cultures to mechanical stretching in the presence of polyclonal anti-human PDGF and anti-human TGF-β antibodies (10 μg/ml). These antibodies are able to recognize all PDGF and TGF-β isoforms, respectively, and it has been shown in separate experiments to completely block the action of PDGF-AA, PDGF-BB or TGF-β1 (data not shown). As can be seen in Fig. 3, these antibodies failed to diminish novel DNA synthesis in PDL fibroblasts exposed to mechanical stretching.

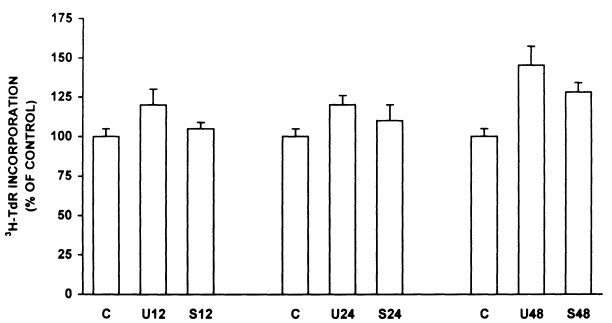

Fig. 4. Mitogenic effect of media conditioned by mechanically-stretched human PDL fibroblasts. Subconfluent cultures of PDL fibroblasts were maintained quiescent in DMEM supplemented with 0.1% FCS. The cultures were subjected to mechanical stretching for 6 h and then were left unstretched. Media conditioned by these cultures were collected after 12, 24 and 48 h (S12, S24 and S48). Media conditioned by unstretched cultures were collected at the same time points, as controls (U12, U24 and U48). All conditioned media were diluted 1:1 with fresh DMEM supplemented with 0.1% FCS, and then added to subconfluent cultures of human PDL fibroblasts remained quiescent in the presence of DMEM supplemented with 0.1% FCS (C); all media contained [^{3}H]thymidine (0.15 μCi/ml). After a 48-h incubation period [^{3}H]thymidine incorporation into newly synthesized DNA was determined. Values represent mean (±S.D.) of three individual experiments performed in triplicate dishes. 100% = 687 cpm (multi-well plates).

Next, we wished to explore if, beyond PDGF and TGF-β, the observed induction of DNA synthesis is due to an autocrine action of released growth factors, in general. To this end, PDL fibroblast cultures were subjected to mechanical stretching for 6 h, and the medium conditioned by these cultures after a total incubation period of 12, 24 and 48 h was collected. At the same time points conditioned media from control (unstretched) cultures were also collected. Fig. 4 shows that media conditioned by stretched cultures were able to induce a small increase in novel DNA synthesis. However, this effect was always less than that elicited by media conditioned from unstretched control cultures. This was also observed when conditioned media from cultures stretched continuously for 24 h or for the total 48-h incubation period were used (data not shown). These results indicate that the increase in DNA synthesis after mechanical stretching of PDL fibroblasts is not due to the production of autocrine growth factors.

Finally, the effect of staurosporine on the stretch-stimulated DNA synthesis was assessed. Staurosporine is a non-specific inhibitor of protein kinases such as A and C (PKA, PKC), which in turn are key elements of the signal transduction pathways initiated by various growth factors [22]. The concentration of staurosporine used (5×10^{-9} M) was the highest that did not induce a direct cytotoxic effect. As shown in Fig. 5, the induction of DNA synthesis after mechanical stretching of PDL fibroblasts, in comparison to control (unstretched) cultures, was practically unaffected in the presence of staurosporine. This corroborates all previous data suggesting that mechanical stretching induces a mitogenic effect on PDL fibroblasts, via a mechanism unrelated to autocrine production of growth factors.

4. Discussion

It has long been recognized that physical forces play a crit-

ical role in the proliferation, differentiation and maintenance of many diverse tissues, particularly those serving a mechanical function. Especially for bone tissue, force application is essential in triggering bone growth and/or remodelling and many treatment rationales are based on this phenomenon [23–25]. In dentofacial orthopedics forces are applied to teeth which in turn are moved through the alveolar bone due to its capacity to remodel. Bone formation occurs in tension sites while bone resorption occurs in compression sites [1]. The exact sequence of events that take place during these processes remains an unresolved issue. Fibroblast cells residing within the PDL, which retain osteoblastic properties and high alkaline phosphatase levels [7], are prominent sensors of this continuous stress and respond in a way culminating in periodontal tissue/alveolar bone regeneration [6]. This process is likely to be influenced, and perhaps modulated, by growth factors. In the present analysis, the response of PDL fibroblasts to mechanical stretching in terms of DNA synthesis induction (as an initial requirement of proliferation) and growth factor involvement was investigated, by employing a well-elaborated, simple in vitro system of stretch application. The stretching forces exerted on the cells in this system are presumably analogous in large degree to the stresses exerted on PDL fibroblasts in vivo [5].

The effect of mechanical stretching on DNA synthesis in PDL fibroblasts isolated from human periodontium, is clearly inductive. This enhancement is obvious even after a brief period (1 h) of exposure to continuous stretch. A similar effect was recorded in other cell types, although different forms of cell stretching were applied [8–11]. More specifically, for vascular smooth muscle cells, this induction in growth after mechanical strain has been ascribed to an autocrine action of PDGF, while basic fibroblast growth factor (bFGF) was not involved [11]. In this vein, the putative involvement of PDGF as well as that of TGF-β1 in stretch-induced DNA synthesis was explored in our study. TGF-β1 was selected because of its pivotal role in bone remodelling [26,27].

PDGF-AA, PDGF-BB and TGF-β1 were found to be strong mitogenic agents for PDL fibroblasts. Moreover, their combined action is synergistic. This synergistic effect of TGF-β1 and PDGF on human PDL fibroblasts has also been reported by Dennison et al. [28], although the PDGF isoform used in their studies is not defined. On the other hand, Matsuda et al. [29] using rat PDL fibroblasts showed that, while PDGF is mitogenic, the action of TGF-β is inhibitory, implying that the effect of this factor might be species-specific. As far as TGF-β1 is concerned, a weak mitogenic effect on human PDL fibroblasts is also reported by Oates et al. [30], whilst in our cell system its action appears to be as strong as that of the potent DNA synthesis inducer PDGF-BB. The mitogenic potency of the two PDGF isoforms (i.e. -AA and -BB) on human PDL fibroblasts has been compared in other studies too: Oates et al. [30] reported that both PDGF-AA and PDGF-BB are strong mitogens. On the contrary, and in accordance to the results presented here, Boyan et al. [31] showed that PDGF-BB is much more effective in stimulating DNA synthesis than PDGF-AA. This observation is consistent with the capacity of PDGF-BB to bind to all three PDGF receptor dimers (i.e. αα, αβ and ββ), whereas PDGF-AA binds only to the αα dimer [14].

Neutralising antibodies to PDGF and TGF-β did not hamper the stretch-induced DNA synthesis in PDL fibroblasts, suggesting that this effect is not dependent on the autocrine action of these two factors. Likewise, Zhuang et al. [10] showed that cyclic, biaxial mechanical strain induces DNA synthesis in human osteoblasts and that this stimulation cannot be blunt by an anti-TGF-β1 antibody. With regard to PDGF, the results presented here are different from those of Wilson et al. [11], who reported that anti-PDGF antibodies reduced the mitogenic effect of mechanical strain in vascular smooth muscle cells. Furthermore, it is hereby demonstrated that conditioned media from stretched cultures of human PDL fibroblasts are less stimulatory than media conditioned from unstretched control cultures, indicating that the increased DNA synthesis after mechanical stretching is not due to an autocrine action of growth factors, in general. This is clearly in contrast to previous data showing that media conditioned from mechanically-stressed cultures of rat vascular smooth muscle cells [11] or rat cardiac myocytes [32] are far more stimulatory than media conditioned from unstressed control cultures, thus suggesting the involvement of an autocrine mechanism in this stimulation. One possible explanation for the discrepancy of these results with those presented here might be the different cell types used (vascular smooth muscle cells or cardiac myocytes vs. PDL fibroblasts), as well as the difference in the species of origin (rat vs. human). In addition, mechanical stretching could alter the production and/or secretion of molecules with mitogenic activity (i.e. growth factors) as a consequence of changes triggered in the human PDL cell membrane.

Our finding that staurosporine is also incapable to inhibit this phenomenon further supports the notion that mechanical stretch-induced DNA synthesis in human PDL fibroblasts is most likely mediated through a mechanism independent of growth factor action. The latter does not conform to the results of Zhuang et al. [10], who reported that neomycin, an inhibitor of inositol phosphate turnover and consequently of diacylglycerol production and PKC activation, blocks mechanical strain-induced DNA synthesis in human osteoblasts.

In summary, this study demonstrates that mechanical strain is an important stimulatory factor in the DNA synthesis of human PDL fibroblasts. Moreover, mechanical strain appears to mediate this effect via a route that is not governed by the autocrine production/release of growth factors. Further work is being carried out utilising our in vitro model to attempt to define biochemically the signalling pathway(s) comprising mechanical load-induced periodontal tissue/alveolar bone regeneration, and to unravel the mechanisms whereby growth factors interact with and/or fine-tune these pathways.

Acknowledgements: We wish to thank our colleague H. Pratsinis for valuable help during the preparation of the manuscript.

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