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Three-Dimensional Collagen Regulates Collagen Gene Expression by a Mechanism That Requires Serine/Threonine Kinases and Is Independent of Mechanical Contraction

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Integrin $\alpha1\beta1$, one of the cellular collagen receptors, can participate in the regulation of collagen accumulation by acting as a negative feedback regulator. The molecular mechanism behind this phenomenon has been unknown. We have plated cells inside three-dimensional collagen and analyzed a set of chemical inhibitors for various signal transduction pathways. Only two wide-spectrum serine/threonine kinase inhibitors, H-7 and iso-H-7 could prevent the down-regulation of $\alpha1$(I) collagen mRNA levels in cells exposed to three-dimensional collagen. In monolayer iso-H-7 slightly down-regulated collagen gene expression, indicating that inside collagen it affected integrin signaling rather than having a direct stimulatory effect on collagen mRNA levels. The effect of iso-H-7 was not dependent on its ability to inhibit protein kinases A, C, or G. H-7 and iso-H-7 could also inhibit collagen gel contraction, but this mechanism was independent of collagen gene regulation. Three-dimensional collagen could also up-regulate the mRNA levels of several matrix metalloproteinases (MMPs) but H-7 and iso-H-7 had no effect on the regulation of MMP genes. Our data indicate that three-dimensional collagenous matrix regulates distinct cellular signaling pathways and that collagen gene regulation is independent of the other effects of the matrix. © 2000 Academic Press

Key Words: **integrins; cell adhesion; collagen; serine/threonine kinases; cell signaling.**

Integrin-type collagen receptors, such as $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$ heterodimers mediate cell adhesion to various collagen types. Like all integrins the collagen receptors are also connected to cellular signal transduction pathways and may regulate cell proliferation, survival, and gene expression. Accumulation of collagenous connective tissue seems to be partially regulated by signaling through $\alpha1\beta1$ and $\alpha2\beta1$ heterodimers. Integrin $\alpha1\beta1$ is a negative feedback regulator of collagen gene expression [1–4], whereas $\alpha2\beta1$ seems to mediate signals leading to collagenase-1 and -3 (matrix metalloproteinases-1 and -13; MMP-1 and -13, respectively) production and collagen degradation [1–5]. Integrin $\alpha2\beta1$ may even be a positive regulator of collagen expression [1, 5]. Induction of MMP-1 by $\alpha2\beta1$ integrin can be prevented by the use of tyrosine kinase inhibitor [6]. Some signal transduction pathways activated by $\alpha2\beta1$ integrin are known. Protein kinase C (PKC) and nuclear factor-κB might be involved in the regulation of MMP-1 [7] and p38 mitogen-activated protein kinase (MAPK) seems to mediate the effect of $\alpha2\beta1$ on MMP-13 and $\alpha1$(I) collagen gene expression [4, 5]. More is known about the signaling via $\alpha1\beta1$ integrin. This receptor is connected to a membrane protein named caveolin-1 and this interaction may lead to activation of tyrosine kinases Fyn and Shc [8, 9]. Shc activates then Ras by the mediation of Grb and Sos. Ras activation leads further to the activation of the Erk MAPK. Tyrosine kinase inhibitors can not however inhibit the $\alpha1\beta1$ integrin dependent down-regulation of collagen synthesis [6] and it is possible that also other signaling pathways participate in collagen regulation. Here we show that two inhibitors for serine-threonine kinases, unlike inhibitors for tyrosine kinases, or protein kinase A (PKA), C (PKC), or G (PKG) antagonists, can prevent cellular signals responsible for collagen feedback regulation.

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PK, protein kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase.

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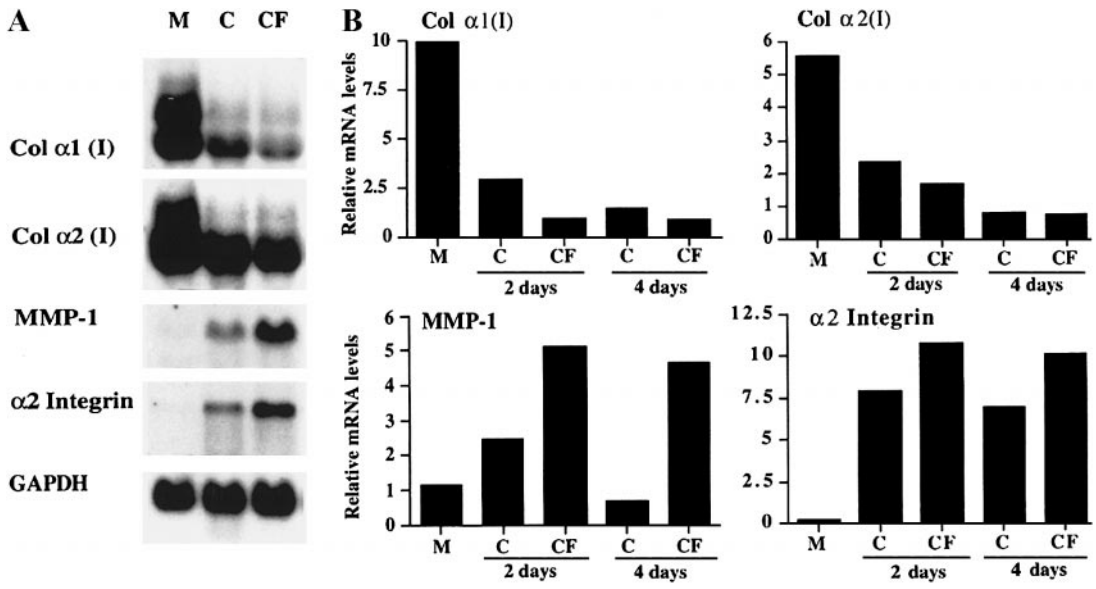

FIG. 1. KHOS-240 cells were cultured for 48 and 98 h on monolayer (M) or inside three-dimensional (3D) collagen gel (500,000 cells/ml), anchored (C), or freely floating (CF). Total mRNA was isolated, separated by electrophoresis, transferred to filters, and the specific mRNA levels were measured with the corresponding cDNA probes. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. (A) Northern blot analysis shows a down-regulation of collagen α1(I) mRNA level when the cells are cultured inside the 3D gel (48 h). C = anchored collagen, CF = freely floating collagen gel. The down-regulation is larger when the gels are floating freely in the medium (CF). (B) The bar graphs show relative levels of collagen α1(I), collagen α2(I), matrix metalloproteinase-1 (MMP-1), and α2 integrin on 48 and 98 h time points. The effect caused by the 3D collagen gel existed on 98 h time point with minor changes.

MATERIALS AND METHODS

Reagents. H-7 and iso-H-7 (inhibitors of several serine/threonine kinases, including PKA, PKC, PKG, and myosine light kinase), KT 5823 (an inhibitor of protein kinase G), KT 5720 (an inhibitor of protein kinase A), Bisindolylmaleimide I (an inhibitor of protein kinase C), and Staurosporine (an inhibitor of several protein kinases, including PKA, PKC, and PKG) were obtained from Calbiochem, La Jolla, CA.

Cell culture. Human osteosarcoma cell line KHOS-240 was obtained from American Type Culture Collection. Cells were maintained in Dulbecco's modification of Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS; Gibco BRL). For collagen gel experiments cells were cultured inside Cellon bovine dermal collagen (Sigma Aldrich). Eight volumes of Cellon was mixed with a 1:1 mixture of 10-fold concentrated medium and 0.1 N NaOH and kept on ice. Cells were detached, counted, and mixed gently into neutralized Cellon solution before they were transferred into 24-well plates (250,000 cells/well). The formation of gels was initiated by incubating the plates at 37°C for 45 min. DMEM supplemented with 10% FCS and inhibitors were then added on gels before detaching the edges of the gels from the sides of the wells. The contraction process was observed daily. Cell culture wells were photographed and the surface areas of the gels were measured from prints.

Northern blots. Total cellular RNA was isolated by using Rneasy kit (Qiagen). Before isolating total RNA from cells inside collagen gels, the gels were briefly treated with 0.5 mg/ml collagenase (type II, Sigma) in phosphate buffered saline (PBS, pH 7.4) with 1 mM $CaCl_2$. Twenty micrograms of total cellular RNA was separated in formaldehyde-containing 1% agarose gels, transferred to nylon membranes (ZETA-probe, Biorad), and hybridized with ^{32}P-labeled (Amersham) cDNA probes for human α1(I) collagen [10], human MMP-1 [11], human MMP-2 [12], human MT-1-MMP [13], tissue inhibitor of metalloproteinases-2 (TIMP-2) [14], human α2 integrin [15], and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a "house keeping" enzyme used as a control) [16]. Autoradiograms were quantified with Microcomputer Imaging Device version M4 (Imaging Research Inc.) and the resulting measurements were corrected for GAPDH mRNA levels.

Flow cytometry. Cells were grown to early confluence, detached with trypsin-EDTA, washed with PBS (pH 7.4) and then incubated with PBS containing 1% FCS for 30 min at 4°C. Cells were collected by centrifugation, exposed to saturating concentration of antibodies against α1 integrin (CD 49a, Serotec, UK) or α2 integrin (12F1) [17] in 1% FCS/PBS for 30 min at +4°C. For labeling cells were incubated with rabbit anti-mouse (1:20 dilution) IgG coupled to fluorescein (DAKO A/S, Glostrup, Denmark) for 30 min at 4°C, washed twice with PBS, and suspended in the same buffer. In order to measure the amount of integrins on cell surfaces, the fluorescent excitation spectra were analyzed by using a FACScan apparatus (Becton Dickinson). Control samples were prepared by treating cells without primary antibodies.

Cell adhesion assays. The coating of a 96-well immunoplate (Maxi Sorp, Nunc, Denmark) was done by exposure to 0.2 ml of phosphate-buffered saline (pH 7.4) containing 4–5 μg/cm^2 type I collagen (from lathyric rat skin, Boehringer Mannheim). Bovine serum albumin was used to measure the non-specific binding. Residual protein absorption sites in all wells were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C. Confluent cell cultures were detached by using 0.01% trypsin and 0.02% EDTA. Trypsin activity was inhibited by washing the cells with 1 mg/ml of soybean trypsin inhibitor (Sigma). 10,000 cells were suspended in DMEM and transferred into each well and incubated at 37°C for 45 min. Non-adherent cells were removed by rinsing the wells with medium. Adherent cells were fixed with 2% paraformaldehyde, stained with 0.5% crystal violet in 20% ethanol and washed with distilled water. The immunoplates were allowed to air-dry, crystal violet in cell layer was dissolved into 10% acetic acid and measured spectrophotometrically at 600 nm with Multiscan Plus (Lab-Systems).

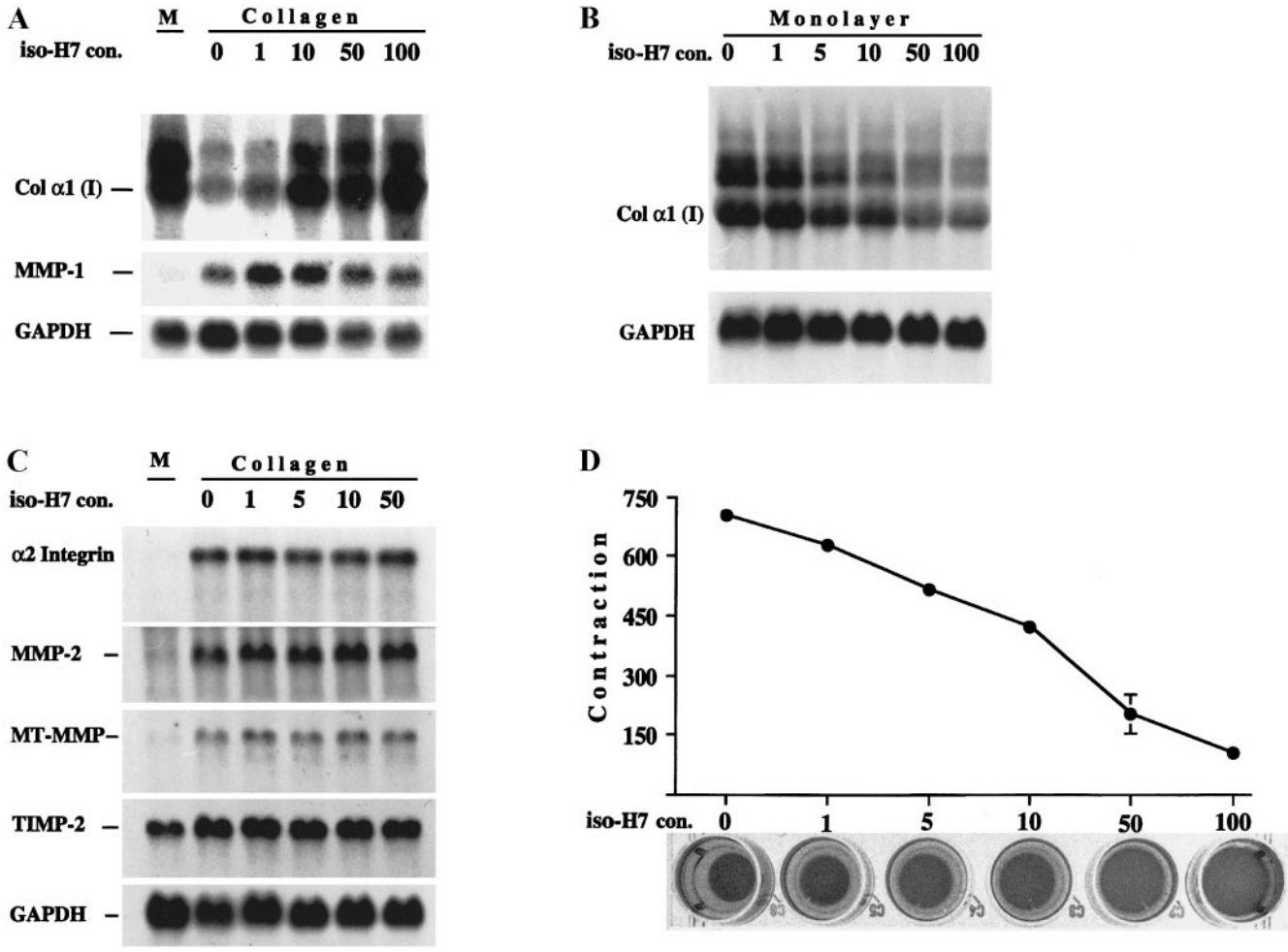

FIG. 2. The effect of serine/threonine kinase inhibitor iso-H-7 on KHOS-240 cells. After two days of incubation in DMEM with iso-H-7 (concentrations shown in the graph), total RNA was isolated from the cells, separated by electrophoresis, and transferred to filters. As shown by (A) iso-H-7 can inhibit the down-regulation of collagen synthesis in cell inside collagen and it has also some positive influence on MMP-1 production. (B) shows that when tested on cells cultured for 48 h in monolayer iso-H-7 has on the contrary, an inhibitory effect on collagen synthesis. The expression levels of α2 integrin, MMP-2, MT-1-MMP, and TIMP-2 mRNAs are shown in (C). (D) KHOS-240 cells were placed inside floating collagen gels (5×10^5/ml) and incubated with different iso-H-7 concentrations for 48 h. A concentration-dependent inhibition of the collagen gel contraction is shown. Data are mean values ± S.D. of four independent experiments. Photograph beneath the graph shows one of these experiments.

RESULTS

Down-Regulation of Collagen Synthesis Is Seen in Cells Inside Collagen Gels, Also When the Gels Are Not Allowed to Contract

Collagen gels can be detached from the walls of the cell culture wells to let the lattices to float freely or the gels can stay anchored [18]. The floating gels will contract in a manner dependent on the presence of $\alpha 2\beta 1$ integrins on cell surface [19, 20]. The two cell culture models, floating and anchored, may have distinct effects on cells. Here human osteosarcoma KHOS-240 cells, known to express both $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins [21], were plated inside collagen gels for 24 or 48 h. mRNA levels of collagen α1(I), collagen α2(I), integrin α2, and MMP-1 were measured by Northern hybridizations (Fig. 1). In accordance with a previous study [20] collagen mRNA levels were down-regulated and α2 integrin and MMP-1 levels were up-regulated. In freely floating gels the effects were systematically slightly larger, however the effects were also seen in anchored gels, with the exception that MMP-1 up-regulation was not seen in day 4 in anchored gels.

Serine/Threonine Kinase Inhibitors Can Prevent the Collagen Gel-Dependent Down-Regulation in Collagen mRNA Levels

We have previously shown that tyrosine kinase inhibitors have no effect on collagen-related down-regulation in collagen gene expression [6]. Now we

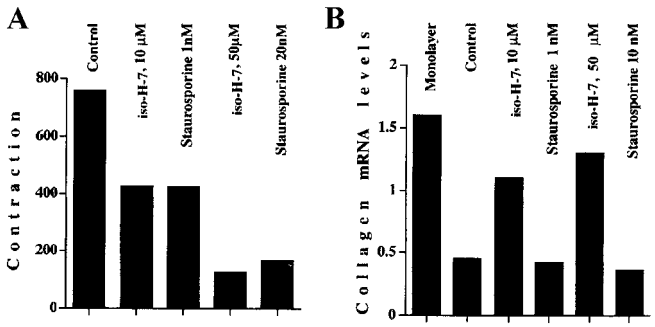

FIG. 3. Effects of serine/threonine kinase inhibitors iso-H-7 and staurosporine (used in the concentrations indicated in the graph) on the contraction of floating collagen gels and collagen α1(I) mRNA levels. Before using these concentrations KHOS-240 cells were tested in monolayer to see whether inhibitors are toxic for the cells. After two days the gels were photographed, total mRNA was isolated, separated by electrophoresis, and transferred to filters and the specific mRNA levels were measured with the corresponding cDNA probes. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The quantity of contraction was measured from the photographs and the results are in (A). Both agents could inhibit the contraction of collagen gel by KHOS-240 cells. On the other hand only iso-H-7 could also inhibit the down-regulation of collagen α1(I) as shown in (B).

tested the effect of wide-spectrum serine/threonine kinase inhibitors, H-7, iso-H-7 and staurosporine. H-7 and iso-H-7 could prevent the reduction in collagen mRNA levels, unlike inhibitors specific for PKA (KT 5720), PKC (Bisindolylmaleimide I), or PKG (KT 5823) (not shown). This suggests that the function of iso-H-7 and H-7 be due to their effects on other serine/threonine kinases. The complete inhibition was seen with iso-H-7 concentration of 10 μM (Fig. 2A). Importantly iso-H-7 had no effect on the increased expression of MMP-1 (Fig. 2A). The effect of iso H-7 was tested also with cells in monolayer and high concentrations (50–100 μM) of iso-H-7 could actually decreased collagen mRNA levels (Fig. 2B) indicating that iso-H-7 is not a direct enhancer of collagen gene expression, but rather effects on collagen receptor signaling as explained in the Discussion. Three dimensional collagen increased the mRNA levels of α2 integrin, MMP-2, MT-MMP, and TIMP-2 but iso-H-7 had no effect on the expression of any of these genes (Fig. 2C). However, it could prevent the contraction of collagen gels in the concentration dependent manner (Fig. 2D). The concentration needed for complete inhibition of contraction (100 μM) was much higher than the concentration needed for inhibition of collagen down-regulation (10 μM).

To confirm that collagen gel contraction and collagen gene down-regulation are independent phenomena we used another kinase inhibitor, staurosporine, and showed that staurosporine concentration (1 nM) had similar effect on contraction than 10 μM iso-H-7 (Fig. 3A) but not on collagen mRNA levels (Fig. 3B). Finally we excluded the possibility that the effect of iso-H-7 could be due to effects on integrin expression or their ability to bind to collagen. This was done by measuring the cell surface α1 and α2 integrin expressions by using flow cytometry (Fig. 4A). Integrin function was tested by a cell adhesion assay (Fig. 4B). Iso-H-7 had no effect on these parameters.

DISCUSSION

Uncontrolled accumulation of collagen is an important clinical problem in various fibrotic diseases. Cytokines and growth factors, such as transforming growth factor-β, are potent regulators of collagen gene expression and therefore potentially involved also in pathological collagen accumulation [22]. Signaling through integrin-type matrix receptors regulates cell proliferation, differentiation, and survival [23]. Integrin-signaling also regulates matrix degradation by controlling the production of MMPs [1–5, 18, 24, 25] and collagen [1–5, 18]. It is possible that these signals also play a role in fibrosis, since for example in scleroderma the expression levels of $\alpha1\beta1$ integrin might be lowered leading to impaired feedback regulation of collagen gene expression [26]. We have recently shown that in mice deficient for α1 integrin, there is increased collagen synthesis in the skin, although concomitantly increased degradation of collagen prevents skin fibrosis [3]. The possibility that $\alpha1\beta1$ integrin signaling may participate in fibrotic processes makes it even more important to know the signaling mechanism of this integrin. Recently, a set of integrins, including $\alpha1\beta1$, was shown

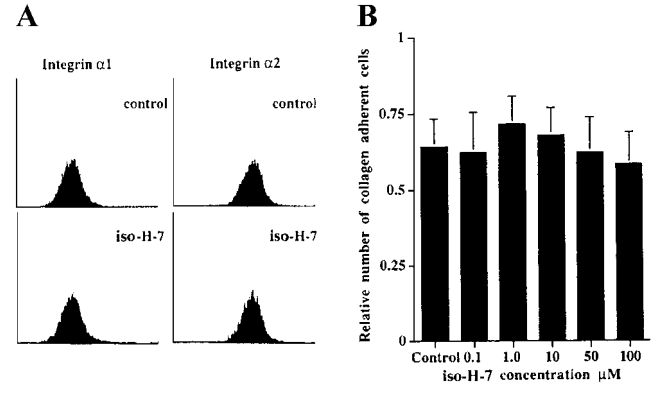

FIG. 4. KHOS-240 cells were grown in a monolayer with or without iso-H-7 (10 μM). After 2 days the cells were harvested and tested by flow cytometry. (A) No change in the expression of the collagen receptors, $\alpha1\beta1$ and $\alpha2\beta1$ integrins, was detected. (B) KHOS-240 cells were detached and suspended in DMEM with different iso-H-7 concentrations and transferred to 96-well immunoplate, 10,000 cells into each well, and incubated for 45 min at 37°C. The number of adherent cells was measured. The bar graph shows mean values ± S.D. (adherent cells/total cell number × 100).

to form a contact with a membrane protein caveolin-1 [8]. Ligation of these integrins leads to activation of Fyn, Shc, Ras, and Erk MAPKs [8]. This is one potential mechanism of collagen regulation. However, the increased Ras and Erk activity in cells results in opposite effects on collagen expression [27], indicating that the regulation mechanism is more complex. We have several reasons to presume that this is not the only mechanism used by $\alpha 1\beta 1$ to down-regulate collagen gene expression. In our previous study various tyrosine kinase inhibitors could not prevent the collagen-related down-regulation in collagen gene expression [6]. Moreover, a specific inhibitor of Erk pathway had no effect on the process. Here, we have tested several inhibitors of various signal transduction pathways and we report that only serine-threonine kinase inhibitors can inhibit collagen-related collagen gene down-regulation in a manner independent of PKA, PKC, and PKG. This observation may guide the future studies on specific signaling pathways to right direction.

Some previous reports have connected the collagen gel contraction process to collagen-related changes in gene expression [28]. Here, the down-regulation of collagen gene expression was mostly independent of the contraction process. In KHOS-240 cells, like also in other osteosarcoma cells, melanoma cells, and fibroblasts, the contraction process is mediated by $\alpha 2\beta 1$ integrin [19, 20, 29]. In certain other cells, including smooth muscle cells and hepatic myofibroblasts, $\alpha 1\beta 1$ integrin may also play some role in contraction [30, 31]. It is possible that in these cell types the mechanism of collagen gene regulation is different, as well.

In addition to collagen also several other genes are differentially regulated inside three-dimensional collagen gel. Several genes in the MMP family, including MMP-1, MMP-2, MMP-13, and MT-1-MMP are activated, as well as tissue inhibitor of metalloproteinases-2 (TIMP-2) [1–4, 32]. Here, the data show that the regulation of these genes is clearly independent of the regulation of collagen expression. Three-dimensional cell culture model itself might change the expression pattern of cellular genes, because cell morphology, organization of cytoskeleton, and adhesion sites may be different. Since integrins also regulate these phenomena some changes in gene expression may be secondary and due to altered morphology rather than direct signaling by a specific integrin.

In summary, previous studies have shown the essential role of $\alpha 1\beta 1$ integrin in the three-dimensional collagen-generated down-regulation of collagen synthesis. Here, we provide evidence that the signaling pathway is dependent on serine/threonine kinases and independent of matrix contraction.

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