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Absence of integrin α1β1 in the mouse causes loss of feedback regulation of collagen synthesis in normal and wounded dermis

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SUMMARY

Integrin α1β1 is a collagen receptor predominantly found in mesenchymal tissues. Mice lacking this receptor are viable. We have previously suggested that α1β1 might participate in the down-regulation of collagen gene expression observed in cells suspended inside collagen gels. The results presented here demonstrate that integrin α1β1 acts as a feedback regulator of collagen synthesis both in vitro and in vivo. Firstly, α1 null animals show a higher rate of collagen synthesis in the dermis in vivo. Secondly, fibroblasts derived from α1 null cutaneous wounds show a reduced sensitivity to collagen gel induced downregulation of collagen mRNA synthesis, as compared to their wild-type counterparts. An increase in collagenase synthesis is also seen in the α1 null dermis and in collagen gel suspended fibroblasts. While dermal thickness is normal in the α1 null animals, an increase is seen in skin thickness of α1 null but not α1 heterozygote animals on a background of collagenase resistant collagen. Increased expression of both collagen and collagenase mRNA are seen in experimental granulation tissue in α1 null animals, but their ultimate accumulation of collagen is normal, probably due to non α1 dependent paracrine regulators of collagen turnover.

Key words: Collagen, Integrin, Dermis, Wound, Collagenase

INTRODUCTION

Integrins are transmembrane receptors for a myriad of extracellular matrix proteins. They provide for cell attachment, motility, and sensing of the extracellular environment. The different integrin heterodimers are well characterised as to their specificity for different ECM components (Hynes, 1992), and some integrins are known to have their affinity for ligand modulated by intracellular events, so called 'inside out' signalling. Responses to the ECM mediated by integrins, so called 'outside in' signalling, such as attachment dependent response to growth factors or avoidance of apoptosis (Ruoslahti and Reed, 1994), have also been demonstrated in a variety of model systems. However, the actual in vivo outside in signalling roles of each individual integrin heterodimer are less well delineated. Gene targeting experiments of the individual subunits have revealed both spectacular and subtle phenotypes (Hynes, 1996). Embryonic lethal phenotypes have usually been associated with specific adhesive deficiencies, such as α5 to fibronectin (Yang et al., 1993) or α4 to VCAM (Yang et al., 1995) both crucial in embryogenesis. The subtle phenotypes found in some other targeting experiments, such as α1 (Gardner et al., 1996) and α7 (Mayer et al., 1997) suggest that a group of integrins are concerned with sensing of the ECM without being absolutely required for structural integrity.

Four integrin heterodimers are known to be collagen receptors; α1β1, α2β1, α3β1 and α10β1 (Gullberg et al., 1992; Yamamoto et al., 1995; Camper et al., 1998). Of these, α2β1 is predominantly epithelial in distribution (Wu and Santoro, 1994). α3β1 is widely distributed and binds a broad range of ligands including fibronectin, laminin 5 (Carter et al., 1991), and collagen, along with cell surface protease systems (Xue et al., 1997). α10β1, recently discovered, is expressed in chondrocytes. α1β1, while having a dynamic pattern of expression in the embryo (Duband et al., 1992), is confined to mesenchymal and endodermal tissues in the adult, notably smooth muscle, fibroblasts, the liver, and microvascular endothelium (Voight et al., 1995; Belkin at al., 1990). All receptors except α10β1 are present in dermal fibroblasts, and all three contribute to collagen I binding (Gardner et al., 1996), although α3β1 may serve only an accessory role (DiPersio et al., 1995). Targeted null mutations for α1 and α3 have been described. α1 null mutant animals show no gross developmental deficits, but null fibroblasts show a specific deficiency in attachment to collagen type IV (Gardner et al., 1996) along with a deficiency in collagen dependent proliferation (Pozzi et al., 1998). α3 null animals are not viable beyond birth, and show specific deficits in epidermal basement membrane formation (DiPersio et al., 1997) and in branching morphogenesis in lung and kidney (Kreidberg et al., 1996).

While fibroblasts from the α1 null animal show a striking absence of adhesion to collagen type IV, they show no appreciable deficit in adhesion to collagen I unless they are functionally deficient in α2 or α3 (Gardner et al., 1996; H. Gardner and A. Pozzi, unpublished observations). The deficiency in collagen IV binding by α1 null fibroblasts was curious, as the location most abundantly supplied with collagen IV is the epidermal basement membrane, and keratinocytes and other epithelial cells are normally devoid of integrin α1. Furthermore, the deficiency in collagen IV binding was not seen in α1 null smooth muscle cells, suggesting that the affinity of another integrin for collagen IV may be modulated by cell type. The α1 null animal did not show any increase in expression of α1β1's closest relative, α2β1 (Gardner et al., 1996; Hughes, 1992).

Several lines of evidence have suggested regulatory roles for α1β1 and α2β1. In osteosarcoma cells the level of cell surface α2β1 integrin correlates with the expression level of native collagenase (MMP1 in humans, or MMP13 in rodents; Shingleton et al., 1996), whereas α1β1 integrin seems to be responsible for downregulation of collagen I mRNA levels seen when the cells are suspended in collagen gels (Riikonen et al., 1995a; Langholz et al., 1995). The use of specific anti-integrin antibodies supports a role for α1β1 as a regulator of collagen synthesis in that a presumably activating anti-α1 antibody accentuates the normal downregulation of fibroblast collagen expression in response to collagen gels (Langholz et al., 1995). Thus, the data available support the role of α1β1 integrin in the regulation of collagen production and suggest that this mechanism may be of importance in fibrotic diseases. Indeed, Ivarsson et al. (1993) have shown that in cell culture, scleroderma skin-derived fibroblasts show a relative decrease in the expression of α1β1 integrin concomitantly with enhanced collagen gene expression and reduced response to extracellular collagen. Thus the α1 null animal may be a model for one step in the pathogenesis of scleroderma, and may be relevant to other fibrotic diseases such as retroperitoneal and radiation induced fibrosis.

At the tissue level, however, growth factors and cytokines are also powerful regulators of collagen accumulation. Transforming growth factors-β (TGFs-β) are often present at the site of inflammation and tissue injury and they are very potent enhancers of collagen synthesis (Massague, 1990). Similarly, cytokines, like interferon-γ and tumor necrosis factor-α, can inhibit collagen gene expression in vivo (Granstein et al., 1987; Rapala et al., 1991). Thus, the importance of integrin-mediated regulation of collagen synthesis and breakdown cannot be interpreted as long as its relation to the cytokine-dependent effects remains unsolved. Here, we have taken a series of complementary approaches to analysis of collagen synthesis and breakdown in the integrin α1 null animal. In vivo analyses include proline labelling of newly synthesized collagen in skin, a genetic test of the role of native collagenase, northern and protein analysis of experimental granuloma tissue over a time course, and histologic evaluation of incisional wounds. We have further studied, ex vivo, primary fibroblast lines derived from embryos and granulation tissue of α1 integrin deficient and control mice.

MATERIALS AND METHODS

Integrin α1 deficient mice

A detailed report about generation and analysis of α1 integrin deficient mice has been published (Gardner et al., 1996). All animals used in the study were α1 nulls or controls on the inbred 129sv/ter background. All experiments used animals between 2 and 5 months of age: for each experiment animals were matched for age and sex.

Cross of the α1 null to the collagenase resistant strain

The collagenase resistant collagen mouse (Liu et al., 1995) has a point mutation in the primary collagenase cleavage site. Animals heterozygous for this mutation develop mild dermal thickening at ages greater than 1 year; homozygotes develop extensive early dermal fibrosis and female infertility after the first litter due to a failure of resolution of uterine implantation sites. Collagenase resistant heterozygotes and α1 nulls were crossed to obtain double heterozygote animals. These were crossed to give the full range of possible genotypes. α1 genotyping was performed as described (Gardner et al., 1996). Collagenase resistant animals were genotyped by restriction digest of the targeted region after nested PCR (protocol a gift of Dr Xin Liu, UCLA), as follows: Round 1; forward primer 5′TGAGACACGAGGCATGGGACC3′, reverse 5′GCATGTCTGA-AGAAGAGGTCT. Round 2; forward 5′GTGAGTATCTGTGG-TTCTGGA3′, reverse 5′CAGGGGGACTGGCTAGGAGGT3′. Each PCR was performed with a hot start, 35 cycles of 94°30s/62°40s/72°120s, in a 50 μl volume with 25 picomoles each primer and 2 mM $MgCl_2$. Template for the first round was 1 μg genomic DNA, and for the second was 1 μl from the first round. 10 μl of the second round product were digested with *Sph*I. The wild-type allele gave an uncut product of 986 bp; while the targeted allele gave digest fragments of 562 and 428 bp. Animals were sacrificed at 6 months of age, and dorsal skin from α1 null/collagenase resistant het and α1 het/collagenase resistant het animals were processed for histology. In order to ensure that sections were matched for their plane of sectioning, skin samples from α1 het and α1 null animals were processed, embedded, and analysed back to back, together, in pairs.

Casein zymography of skin explants

3× 3 cm segments of depilated dorsal skin from two wild-type and two α1 null adult animals were cut into 2 mm square pieces and incubated in 10%FCS for 48 hours in a 25 cm flask to allow fragment attachment. Fragments were washed repeatedly with PBS, and 5 ml DFCI (Band and Sager, 1989) was added to the flask. After 6 or 12 hours the medium was recovered, dialyzed against water for 24 hours, lyophilized, and resuspended in 100 μl Laemmli's sample buffer without mercaptoethanol or Bromophenol Blue. Protein concentration was measured by micro-BCA (Amersham). 60 μg total proteins were loaded for zymography on 10% SDS-PAGE containing 1 mg/ml casein (Miyazaki et al., 1990), and run at 10 mA for 3 hours in non-reducing conditions. Gel proteins were renatured in 50 mM Tris pH 7.5/0.1 M NaCl/2.5% Triton X-100 for 2 hours at room temperature, washed with water, and then incubated for 17 hours in 50 mM Tris/10 mM $CaCl_2$/0.02% NaAzide. Gels were stained with Coomassie blue and destained in 5% acetic acid/10% methanol. Control gels were incubated in the presence of 10 mM EDTA instead of calcium, to rule out serine protease activity.

Experimental granulation tissue in mice

A viscose cellulose sponge (Cellomeda Oy, Turku, Finland) was used as an inductive matrix for granulation tissue (Niinikoski et al., 1971) The sponges were cut to 5 mm × 5 mm × 10 mm pieces (weight about 15 mg), decontaminated by boiling for 30 minutes in physiological saline and implanted under the back skin of anesthetized animals using an aseptic technique. After 4, 7, 14, or 21 days the animals were killed and sponges analysed for dry weight, nitrogen content (Minari

and Zilversmit, 1963) and hydroxyproline content (Woessner, 1961). Histological samples were prepared and total RNA was isolated for northern blot analyses.

Granulation tissue derived primary cell lines

Two week old cellulose sponge induced granulation tissue masses were cut into small pieces, which were air dried on plastic cell culture dishes, and medium (DMEM/10% fetal calf serum) was added. Cells were allowed to migrate out of sponges for two weeks before they were detached by trypsin and subcultured. Cells were suspended in floating, nonstressed collagen gels as previously described (Riikonen et al., 1995a,b).

Experimental cutaneous wounds

Animals were anaesthetized with isoflurane and subjected to two 1 cm long full thickness transverse skin incisions on the dorsum. Incisions were immediately closed with three interrupted 5/0 prolene sutures. Animals were killed 12 days postoperatively, and the dorsal skin excised and fixed in neutral buffered 10% formalin. Wounds were embedded in paraffin and sectioned in the saggital plane. Sections were stained with H&E and Masson's Trichrome. Normal skin samples were processed similarly.

Incorporation of ^{3}H-proline into collagen in vivo

Analysis of dermal ^{3}H-proline incorporation was carried out essentially as described by Buck et al. (1996). Briefly, five wild-type and five α1-null mice (129sv/ter male, 60 days old) were given intraperitoneal injections of 50 μCi [5-^{3}H] proline (Amersham) in 0.2 ml of sterile PBS. After 3 hours, the animals were killed, and completely shaved. The truncal skin was then removed and placed immediately in ice-cold buffer, containing 50 mM Tris-HCl, pH 7.5, 15 mM EDTA, 1 M NaCl, and 1 mM phenylmethylsulfonyl fluoride. The skin was homogenized for 2 minutes at high speed with a tissue homogenizer (Tissue Tearor, Fisher). A portion of the skin homogenates was used to determine the total amount of proteins as follows: skin proteins were precipitated from 1/10 of the total homogenate three times with 65% ethanol. The precipitates, collected at 3000 ***g*** for 10 minutes at 4°C, were solubilized with 0.2 N NaOH. The remainder of the skin homogenate was used to purify collagen, as described by Buck et al. (1996). Briefly, homogenates were digested with pepsin (10 mg/g) in 0.5 N acetic acid at 4°C for 6 hours, clarified, and the supernatants from two rounds of digestion were neutralised and precipitated with 176 mg/ml $(NH_4)2SO_4$. Precipitates were washed with 70% ethanol and redissolved in 0.1 N NaOH. Protein concentrations were measured with the Micro BCA protein assay (Pierce, Rockford, IL), standardised with BSA for the total protein measurements and Vitrogen 100 for the collagen measurements. Equal amounts of total proteins and collagen were used to determine incorporated radioactivity by liquid scintillation counter (LS 5801, Beckman Instruments Inc,). The specific activity of dermal collagen was expressed as a proportion of specific activity of total proteins to normalize for variations in bioavailability of the injections.

Northern blot hybridization

Total RNA was isolated by using the guanidium thiocyanate/CsCl method (Chirgwin et al., 1979). Skin samples were snap frozen and ground in liquid nitrogen prior to guanidium lysis. For cells embedded in 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 $CaCl_2$. 10 μg of total cellular RNA was separated in formaldehyde-containing 1% agarose gels, transferred to nylon membranes (ZETA-probe, Bio-Rad), and hybridized with ^{32}P labeled (Amersham) cDNAs for mouse α1(I) and α1(III) collagen (Metsaranta et al., 1991) and mouse MMP13 (gift of P. Soloway). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fort et al., 1985) or mouse β-2 tubulin (gift of K. Sullivan) were used as probes for loading controls. Autoradiograms were quantified with Microcomputer Imaging Device version M4 (Imaging Research Inc.), and normalised to GAPDH levels.

RESULTS

α1 null dermis shows increased steady state levels of collagen synthesis

In order to determine whether the level of collagen production was altered in the α1 null skin, we undertook an in vivo labelling experiment (Fig. 1). 5 α1 null and 5 control animals were injected intraperitoneally with 50 μCi ^{3}H-proline, and sacrificed after 3 hours. Total truncal skin was excised, and the specific activity of extracted collagen was determined relative to the specific activity of total skin proteins, according to the method of Buck et al. (1996). Proline incorporation into collagen relative to total proteins was increased 20% in the α1 null animals, indicating higher steady state levels of collagen synthesis in the null animals. This suggests that α1β1 normally provides inhibitory feedback upon collagen I synthesis. Northern analysis showed that α1(I) collagen mRNA levels varied widely between sites in intact skin (not shown), and was unhelpful in determining overall levels of synthesis.

Dermal thickness in α1 null animals is not altered, probably due to increases in collagenase levels

Despite the increased levels of collagen synthesis, histological sections of α1 null and control skin showed no differences in dermal thickness (Fig. 2A, upper). However, northern blot analysis of skin samples showed increased levels of collagenase mRNA in α1 null animals when compared to the wild-type animals (Fig. 2A, middle). Furthermore, explants of α1 null skin produced native collagenase activity not seen in

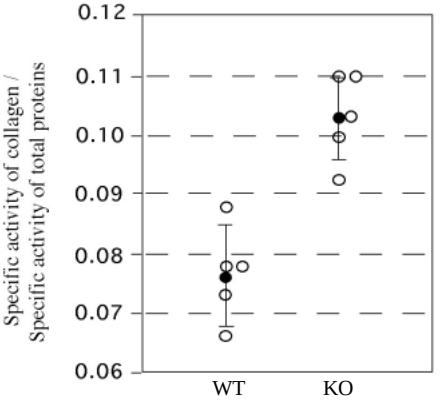

Fig. 1. Specific in vivo incorporation of tritiated proline into collagen by wild-type and α1 null animals. Five wild-type and five α1 null adult male 129sv mice were injected intraperitoneally with tritiated proline three hours prior to sacrifice. Truncal skin was removed and homogenized. Portions of the homogenates were used to prepare total proteins, and the remainder subjected to collagen preparation by an acid extraction protocol (see Materials and Methods). Results are expressed as specific activity of extracted collagen/specific activity of total proteins. Circles indicate results from individual animals, and black dot and bars indicate mean and standard deviation. *P*=0.0007 by two tailed *t*-test assuming equal variances.

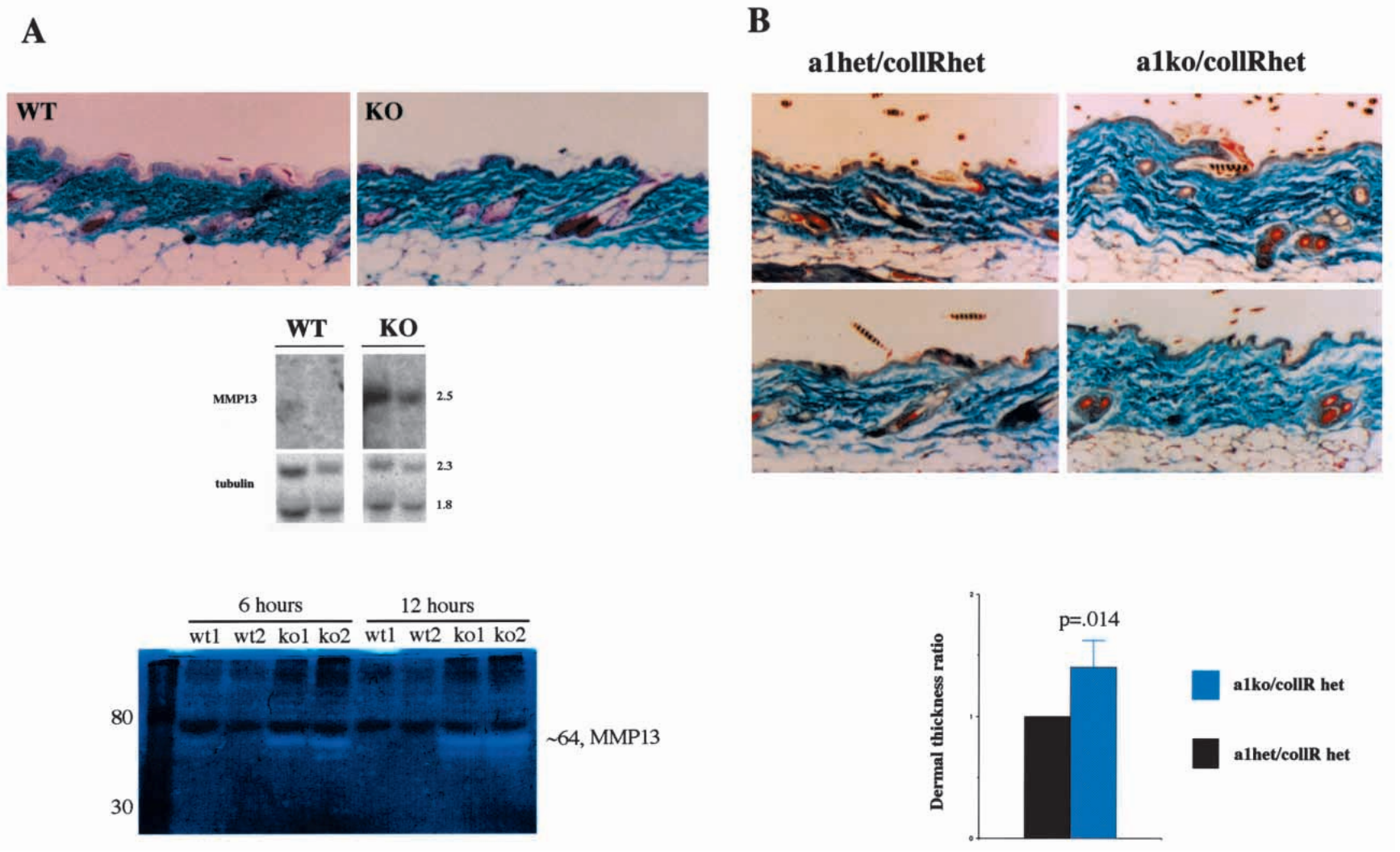

Fig. 2. Skin and collagen turnover. (A) Upper: side to side comparisons of wild-type (WT) and and α1 null (KO) dorsal skin from age matched 129sv males. Note similar thickness of the dermis in both specimens. Paraffin sections, Masson's trichrome, objective magnification ×10. (A) Middle: northern analysis of whole, unwounded, dorsal skin from 2 wild-type (WT) and 2 α1 null (KO) animals, probed for murine collagenase 1 (MMP13) and tubulin. The α1 null animals show detectable collagenase mRNA, while the wild type do not. (A) Lower: casein zymogram of full thickness skin explants from two wild-type and two 2 α1 null animals. Skin fragments were incubated in the presence of serum free DFCI medium for 6 or 12 hours. Medium was recovered for zymography as described in Materials and Methods. Numbers indicate sizes in kilodaltons. A 62-64 kDa lucent band is present in the 2 α1 null samples (ko) and is not seen in the wild type (wt). The band did not appear in zymograms incubated in the presence of EDTA (not shown), and is therefore consistent with MMP13, rather than a serine protease. (B) Upper: comparison of dorsal skin samples from two α1 heterozygous (left) and two α1 null (right) 6 month old females on a heterozygous collagenase-resistant collagen background. The upper panels show one pair of samples embedded together, and the lower panels a second pair. Note the increase in thickness in the α1 null/collR het samples compared to the α1 heterozygous/coll R het samples. Masson's trichrome, objective magnification ×10. (B) Lower: mean and standard deviation of dermal thickness of six α1 null samples expressed relative to the thickness of their respective heterozygous pair.

wild type, as assayed by casein zymogram (Fig. 2A, lower). Thus, even as collagen I synthesis is increased in the α1 null, expression of the major native collagenase is also increased. This would appear to prevent the increase in collagen synthesis from causing an increase in dermal thickness. To test this possibility, we crossed the α1 null to the collagenase resistant collagen mouse (Liu et al., 1995), which is engineered in the COL1A1 gene to give a point mutation in the primary collagenase cleavage site. Heterozygotes for this mutation have essentially normal skin thickness at ages under 1 year, while homozygotes develop dramatic skin thickening early in life. Analysis of six pairs of dorsal skin samples from age and sex matched α1 heterozygotes and α1 nulls on a collagenase resistant heterozygous background showed a striking increase in skin thickness in the α1 nulls (Fig. 2B). Thus, when the effects of collagenase are reduced in vivo by partially resistant collagen, the increased collagen synthesis in the α1 null results in skin thickening.

α1 deficient embryonic and granulation tissue derived fibroblasts show blunted responses to exogenous collagen in vitro

Granulation tissue cell lines were established by enzymatic digestion of 14 day old viscose cellulose sponge induced experimental granulomas. Embryonic lines were derived by trypsinization of eviscerated E14.5 embryos. Cell lines were derived from both α1 integrin null and control animals. The basal level of α1(I) collagen mRNA was studied in cell lines cultured on normal tissue culture plastic in the presence of 10% fetal calf serum. In repeated experiments with cell lines from different animals no differences were detected between wild-type and α1 null lines (Fig. 3A). Similarly there were no differences in α1(III) collagen mRNA levels (not shown). mRNA for murine collagenase was undetectable in all cell lines (not shown). When the same cell lines were cultured inside three dimensional collagen for two to five days, α1(I) collagen mRNA levels were remarkably lower in control mice-derived cell lines than in α1 null cell lines (Fig. 3A, Fig. 4). This indicates that the collagen gel-dependent downregulation of α1(I) collagen mRNA levels requires, at least in part, the presence of α1 integrin. Collagen α1(III) mRNA levels were slightly higher in α1 null cell lines, as well, although the difference was less pronounced than that seen in α1(I) collagen mRNA levels (Fig. 3B). By day 5, while collagen expression was still higher in α1 nulls than wild type, the α1 null cells also showed an appreciable increase in collagenase expression compared to wild type (Fig. 3C). No differences in collagen gel contraction were observed between wild-type and α1 null lines.

Increased collagen gene expression at an early stage of granulation tissue formation does not affect the normal development of experimental granulation tissue

The formation of new connective tissue in mice was studied by implanting viscose cellulose sponges subcutaneously. The overall histology of the granulation tissue and capillary ingrowth appeared similar in both α1 null and control animals (not shown). Dry weight and total protein content of the granulation tissue masses increased in the same way regardless of the presence or absence of α1 integrin (not shown). Collagen gene expression was studied at different stages of developing granulation tissue by northern blot hybridizations. Collagen α1(I) mRNA levels were compared to levels of GAPDH mRNA and to rRNA. At an early stage (day 4) of granulation tissue development, α1 deficient animals showed 2.1-fold higher α1(I) collagen mRNA levels than the controls (Fig. 5A,C), consistent with the findings in granulation tissue fibroblasts suspended in collagen gels. A very slight increase in α1(III) mRNA was also present (Fig. 5A). Histological

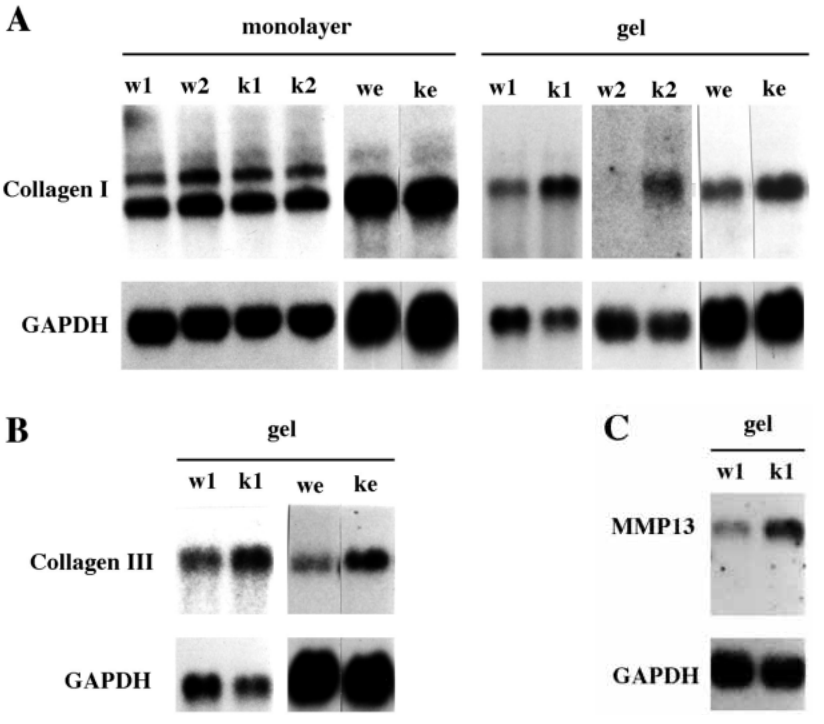

Fig. 3. mRNA levels in primary granulation tissue cells in monolayer culture derived from two wild-type (w1,w2) and two αl integrin deficient mice (k1,k2), and in embryonic fibroblasts from a wild-type (we) and an α1 null (ke) embryo. (A) Collagen α1(I) in cells in monolayer culture (monolayer) or from the same cells suspended in three-dimensional collagen gels (gel) for two (w1, we, k1 and ke) or five (w2 and k2) days. (B) Collagen α1(III) mRNA from cells suspended in gels for 2 days. (C) Murine collagenase 1 (MMP13) mRNA levels from cells suspended in collagen gels for 5 days. Total RNA was isolated from cells and separated in formaldehyde-containing agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labeled cDNAs for mouse α1(I) collagen, α1(III) collagen, rat collagenase (MMP13), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a 'house keeping' enzyme used as a control) probes.

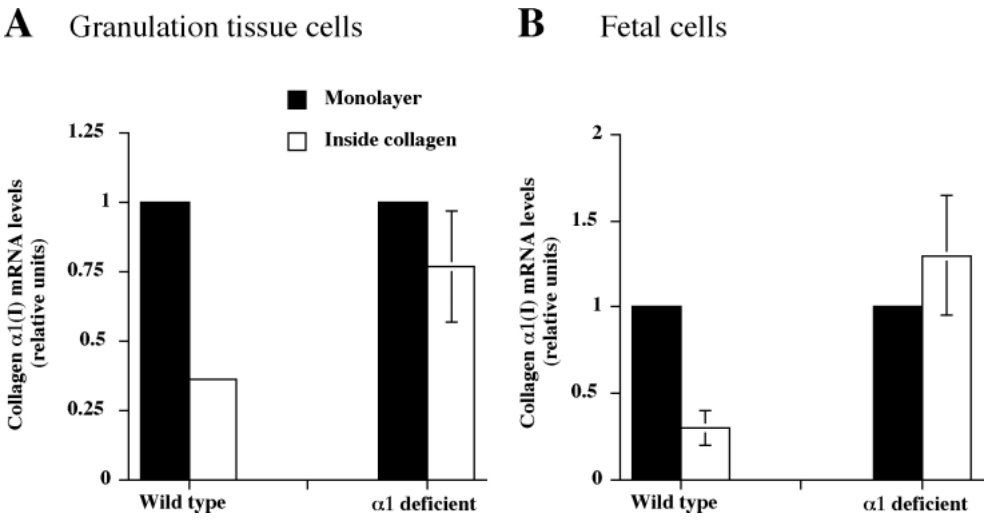

Fig. 4. α1(I) collagen mRNA levels in wild-type (Wild type) and α1 integrin null (α1 deficient) granulation tissue cells (A) and fetal (B) cells in monolayer and inside collagen. Cells were cultured in monolayer or inside collagen for 2 days before recovery of mRNA and northern analysis as described in Fig. 3. Collagen mRNA levels were normalised with the GAPDH mRNA level, and the level in gels expressed as a ratio of the level in monolayer. (A) Two experiments done with two independent isolates of α1 integrin deficient granulation tissue cells (mean and range) compared to a wild-type granulation tissue line. (B) Four independent experiments done with two α1 null fetal cell lines (mean and s.d.) compared to two independent experiments done with two wild-type fetal lines (mean and range).

examination of day 4 granulation tissue indicated that both α1 deficient and control animals had some cells throughout the sponge, but more than 90% of cells were within 1 mm of the edge of the sponge (not shown). However, the relative levels of α1(I) collagen RNA increased about 10-fold from day 4 to day 21 in control animals and to a similar maximum in α1 null animals (Fig. 5B), and at these later stages of granulation tissue formation (days 7, 14 and 21) no major differences were detected in the collagen mRNA levels between wild-type and α1 null animals. Accumulation of collagen was studied by measuring the amount of hydroxyproline in granulation tissue (Fig. 6A). While initially the increase in the amount of collagen followed the increase in collagen mRNA levels, by day 21 the collagen content of the granulomas had decreased while the collagen mRNA levels were still high, suggesting that collagen degradation was also occurring. Indeed, by day 7, collagenase mRNA, already detectable in wt and ko granulation tissue, reached approximately twofold higher levels in the α1 nulls

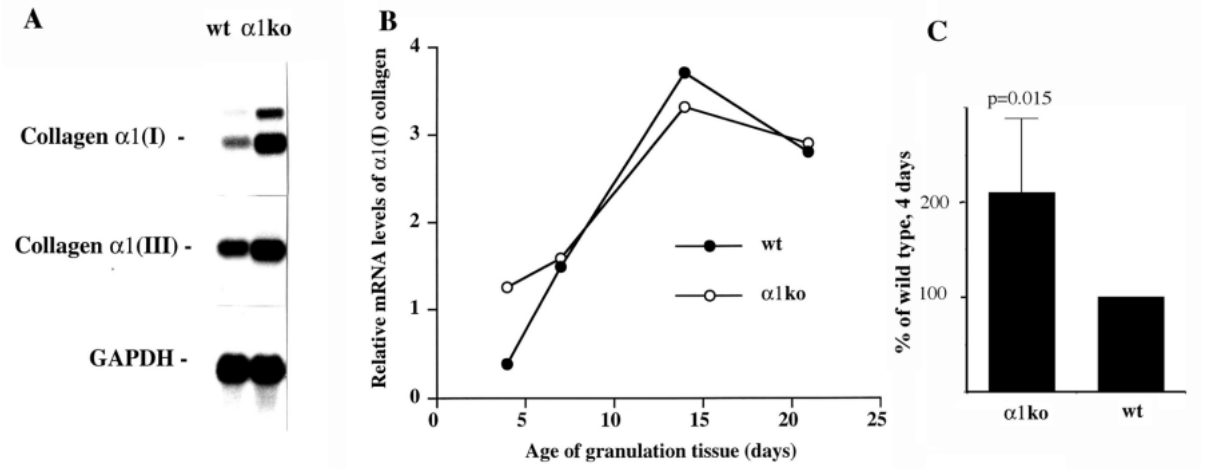

Fig. 5. Collagen α1(I) mRNA levels in different stages of granulation tissue formation. (A) Representative experiment showing α1(I) collagen mRNA and α1(III) collagen mRNA levels at day 4 in wild-type and α1 integrin deficient mice. Total RNA from four granulation tissue masses (from four separate animals) was pooled together for northern blot analysis. (B) The remarkable increase seen in relative α1(I) collagen mRNA levels seen during the first three weeks of granulation tissue formation. (C) Mean and standard deviation of collagen α1(I) mRNA in α1 null granulomas expressed as a percentage of that seen in wild type from three independent experiments at day 4. Total RNA was isolated from granulomas and separated in formaldehyde-containing agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labeled cDNAs for mouse α1(I) collagen and rat glyceraldehyde-3-phosphate dehydrogenase. Autoradiograms were quantified with an image analyser and the resultant measurements corrected for GAPDH mRNA levels. For each data point total RNA from four granulation tissue masses (from four separate animals) was pooled together for northern blot analysis.

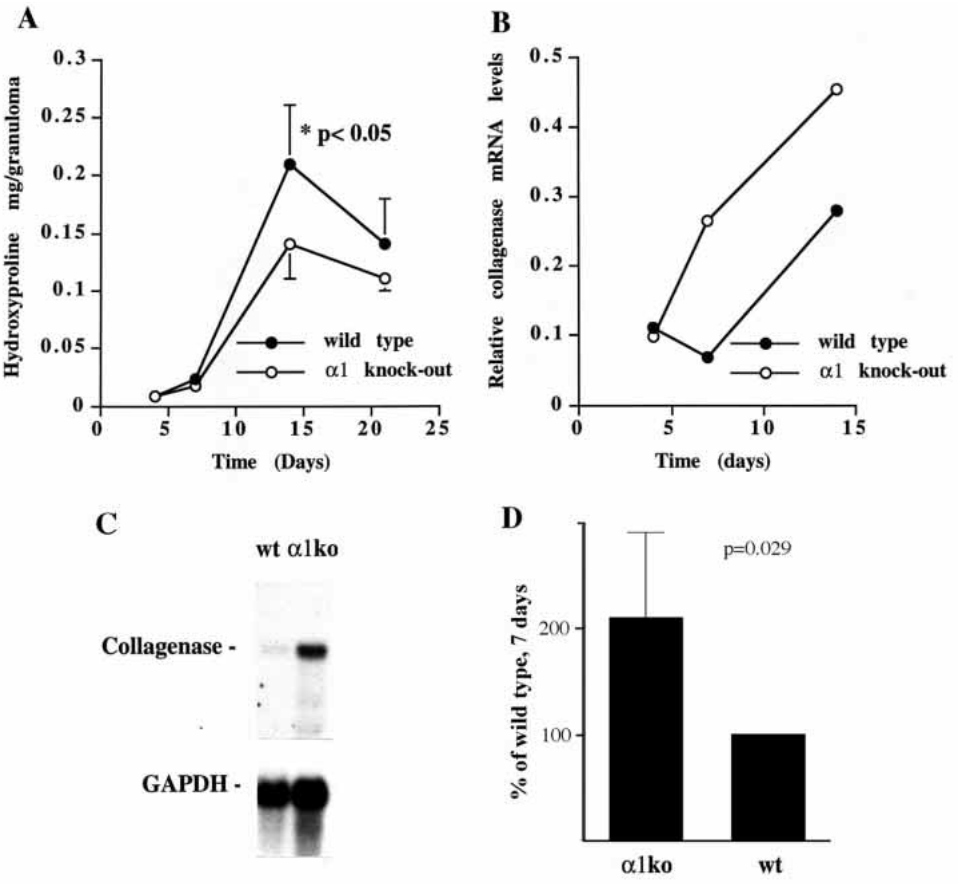

Fig. 6. (A) Accumulation of collagen to the experimental granulation tissue in wild-type and α1 integrin deficient mice. Viscose cellulose sponges were implanted subcutaneously into the animals. The collagen content, measured as the amount of hydroxyproline, was analyzed at indicated time points. The data represents mean and standard deviation of six measurements from separate animals. The difference at day 14 was statistically significant by 2 tailed *t*-test assuming equal variances, with a *P*-value of 0.049. (B) Collagenase (MMP-13) mRNA levels in granulation tissues. Quantification of day 4, 7, and 14 northern blots. (C) Example northern blot of day 7 granulation tissue samples. (D) Mean and standard deviation of collagenase mRNA in α1 null granulation tissue masses expressed as a percentage of that seen in wild type from three independent experiments at day 7. Total RNA was isolated from granulomas and separated in formaldehyde-containing agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labeled cDNAs for mouse collagenase (MMP13) and rat glyceraldehyde-3-phosphate dehydrogenase. Autoradiograms were quantified with an image analyser and the resulted measures were corrected for GAPDH mRNA levels. For each data point the total RNA from four granulation tissue masses (from four separate animals) were pooled together for northern blot analysis.

(Fig. 6B,C,D) and the difference persisted to day 14. The collagen content of granulomas in α1 integrin null animals was slightly lower than in controls (Fig. 6A), indicating that the higher collagen mRNA levels detected at early stage of the granulation tissue formation did not cause an increase in the ultimate accumulation of collagen, or in the temporal pattern of the accumulation.

Incisional wounds showed altered morphology in α1 null animals

We determined whether the alterations in collagen metabolism seen in collagen gels and sponges were reflected in vivo by performing full thickness incisional wounds. Trichrome staining of wounds at day 7 (not shown) revealed similar morphologies in wild-type and α1 null animals, with a similar pattern of granulation tissue infiltration and vascularisation at the site of incision. By day 12, however, the distribution of collagen fibrils appeared to be altered in α1 null wounds at the site of incision as revealed by trichrome (Fig. 7) and van Gieson's (not shown) stains; in the wild-type animals, the collagen was distributed evenly between the granulation tissue fibroblasts. In the α1 null animals, however, collagen staining showed a variegated, nodular pattern. Small darkly stained areas alternated with nearly lucent areas, suggestive of an exaggeration in the normal process of collagen synthesis and remodelling. This appearance is consistent with the findings in granulation tissue fibroblasts, of both increased synthesis and breakdown of collagen.

DISCUSSION

Integrin α1β1 mediates feedback regulation of collagen synthesis

The interaction of integrins with matrix and with intracellular components may mediate structural strength, and continuity between the cytoskeleton and ECM, or may be responsible for transmission of other signals from inside out or outside in.

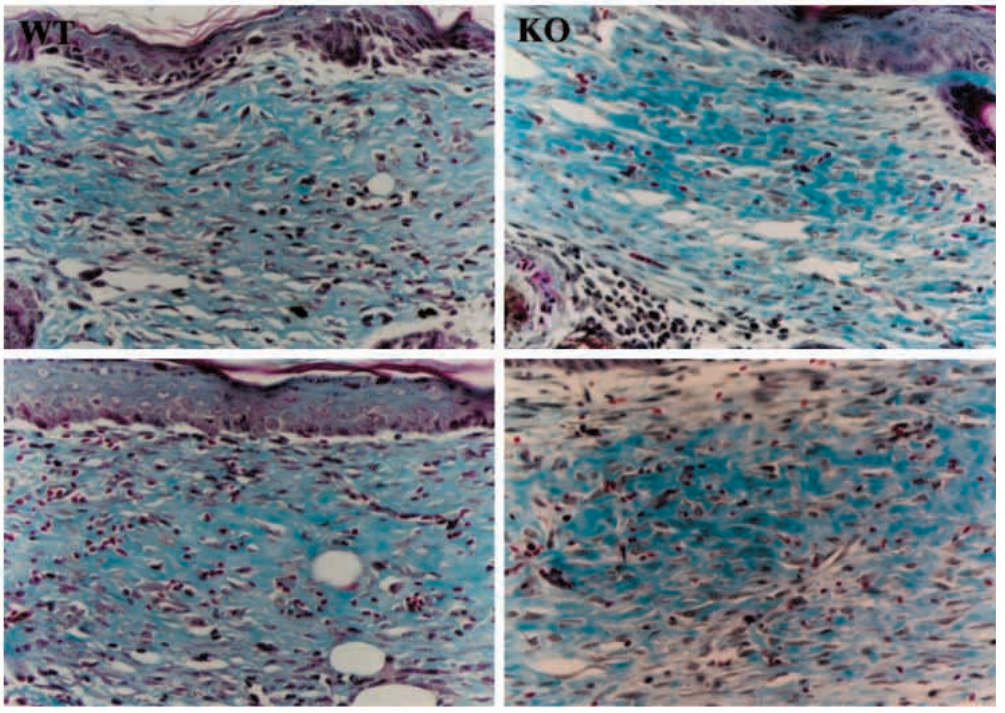

Fig. 7. Masson's trichrome stain of 12 day wounds from 2 wild-type (left, WT) and two α1 null (right, KO) animals. Photographs show the dermis at the point of the incision. The α1 null animals show marked variegation in trichrome staining intensity of the fibrillar collagen, suggestive of irregular deposition or organisation in the wound. Objective magnification ×40.

Integrin α1β1 is one of two major collagen receptors, and is the sole collagen receptor of some mesenchymal tissues, notably smooth muscle. It was therefore a striking result that the α1 null animal had no obvious structural deficit, and suggested that the major role of the molecule is in signalling. In this regard, studies suggesting a role for α1β1 in the regulation of collagen production offered a clue as to the function of this integrin. The idea of α1β1 integrin being a regulator of collagen synthesis has been based on three observations: skin fibroblasts from scleroderma patients show upregulated collagen synthesis and a concomitant downregulation of α1 expression (Ivarsson et al., 1993). An α1 integrin negative osteosaroma cell line failed to downregulate collagen synthesis in collagen gels while α1 positive lines were able to do so (Riikonen et al., 1995a). Lastly, one anti-α1 integrin antibody tested, when used in combination with an anti-β1 antibody, was able to accentuate the normal downregulation of collagen synthesis by normal fibroblasts in collagen gels (Langholz et al., 1995).

We now demonstrate that α1β1 is a feedback inhibitor of collagen synthesis in vivo using integrin α1 null mice. α1 null animals show raised steady state levels of collagen synthesis, and α1 null fibroblasts are deficient in downregulation of collagen synthesis when exposed to collagen gels in assays similar to those used in previously published experiments (Langholz et al., 1995; Ivarsson et al., 1993; Broberg and Heino, 1996). Increased levels of collagen synthesis do not, however, cause an increase in dermal thickness, due to an increase in the expression of collagenase. The effects of the collagenase can be partially overcome, in vivo, by a cross of the α1 null into a collagenase-resistant collagen background, and on this background an increase in skin thickness is evident. The preponderance of evidence suggests that it is α2β1 activation which regulates collagenase expression (Riikonen et al., 1995a,b; Langholz et al., 1995) and there is no published evidence to suggest that collagenase levels are directly regulated by integrin α1β1. Therefore, the activation of collagenase expression in α1 null animals may well be due to increased levels of collagen interacting with the normal levels of α2β1 receptor.

The mechanism of collagen regulation by integrin α1β1

Both α1β1 and α2β1 integrins might be important in cell migration and organization of collagen fibrils. However, their signal transduction function seems to be different. α1β1 is distinct from α2β1 in being able to recruit Shc and activate MAP kinase (Pozzi et al., 1998). MAP kinases and AP 1 transcription factors have been implicated in the regulation of collagen genes (Davis et al., 1996; Chung et al., 1996), suggesting a molecular route of connection between α1β1 and collagen. However, tyrosine kinase inhibitors which prevent the signaling associated with α2β1 integrin do not have an effect on collagen gel-dependent downregulation of collagen α1(I) RNA levels (Broberg and Heino, 1996), and thus α1β1 regulation of collagen gene expression may in fact be mediated via a tyrosine kinase independent pathway.

Collagen receptors and wound healing

While the changes in collagen turnover in unwounded α1 null skin appear to be straightforward, the consequences of α1 deficiency on wound healing are more complicated. The process of wound healing includes, in addition to epithelial cell proliferation and migration, the activation of stromal connective tissue, the formation of granulation tissue, and the generation of collagenous scar. The important role of integrins in different stages of epithelial cell migration is well studied (Clark, 1990; Larjava et al., 1993) whereas the role of integrins in scar formation is less well known. Of the collagen receptors, α2β1 has been subject to some analysis. While hardly

detectable in normal pig skin and in wound granulation tissue when the matrix is still rich in fibronectin, its expression is strongly upregulated with the formation of collagenous scar and the beginning of the scar contraction (Xu et al., 1996). This is in accordance with observations suggesting that α2β1 integrin is the receptor required for collagen gel contraction (Riikonen et al., 1995a; Schiro et al., 1991; Klein et al., 1991). The role of α1β1 integrin has not hitherto been clearly established. However, growth factors like platelet-derived growth factor stimulate α2β1 integrin expression (Xu et al., 1996; Ahlen and Rubin, 1994), and TGFβ stimulates both α2β1 and α1β1 in cell culture (Riikonen et al., 1995b; Heino et al., 1989) and probably also in healing tissue.

As well as affecting the expression of integrins, various cytokines including TGFβ have direct effects upon collagen synthesis, and possibly other events in collagenous scar formation. Experiments done with isolated cells in culture do not take into account other potential regulatory factors which are present in tissues and might be more potent regulators of collagen biosynthesis and breakdown than extracellular matrix alone. Here, we have taken an approach to study this question, using the well characterized model of viscose cellulose sponge implants to test connective tissue activation. In both wild-type and α1 null mice, the overall kinetics of collagen accumulation were similar: mRNA levels began to rise at day 4, followed by detectable increases in collagen accumulation in the sponge by day 7. Collagen levels peaked at day 14 and then decreased by day 21, despite a persisting rise in mRNA levels. Thus, it is likely that other factors than extracellular matrix and matrix receptors regulate the synthesis and accumulation of collagen. TGFβ, a strong inducer of collagen synthesis, is the likely cytokine to override the feedback functions of α1, making the effect of the presence of α1 negligible in this context. Furthermore, as the amount of collagen in sponges decreased between days 14 and 21, while the corresponding mRNA levels were still high, collagen degradation may be, ultimately, the most important regulator of collagen accumulation in granulation tissue. However, despite the general similarity in the 'envelope' of collagen accumulation in wild-type and α1 null granulation tissue, the data assembled here suggest that overall collagen turnover is increased in wounds in α1 null animals. It would seem that the increase in collagen synthesis due to α1 deficiency is more than compensated for by the increase in collagenase activity also present, hence the ultimate reduction in hydroxyproline incorporation into granulation tissue in the α1 null animal. While we have not observed alterations in the structure of dermal collagen in the unwounded state, the histology of the healing wound may highlight histologically imperceptible alterations present in unwounded α1 null dermis. We have observed anecdotally that α1 null skin is weaker than wild type, and that exodus of fibroblasts from dermal explants is faster in the null animal. Both of these phenomena would be accounted for by an increase in collagenase expression and an alteration in the collagen microarchitecture. We cannot ignore the possibility that we have ablated one arm of a finely tuned mechanism, and that the remaining α2β1 integrin, while expressed at normal levels, is somehow left uncontrolled. In this way, it is possible that α2 mediated signalling may make a large contribution to the witnessed phenotype.

The nature of collagen mediated feedback

While these and previous studies offer an appealing summary of the roles of integrins α1β1 and α2β1 in collagen metabolism, with α1β1 providing feedback inhibition on collagen synthesis, and α2β1 providing positive regulation of collagenase, there remains some mystery as to the nature of the collagen-receptor interaction that occurs. In the tissue culture system it is possible that there is simply an increase in ligand available when cells are transferred from monolayer to collagen gel. We now show that α1β1 dependent downregulation of collagen synthesis also takes place in normal dermis, where most of the collagen would be expected to be in highly organised fibrils (Prockop and Kivirikko, 1984) and thus likely largely inaccessible to receptor. In this context, a simple extracellular 'concentration' of collagen I is unlikely to be meaningful. Instead it is likely that either α1β1 and α2β1 occupancy is altered by very localized concentrations of newly synthesized collagen, or that the orientation of adjacent fibrils might alter clustering of bound integrins. In either case, more sophisticated in vitro studies comparing the effects of linear and crosslinked collagen on α1β1 and α2β1 mediated effects are warranted in the future.

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