Downregulation of Tenascin Expression by Glucocorticoids in Bone Marrow Stromal Cells and in Fibroblasts

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Abstract. Tenascin, a predominantly mesenchymal extracellular matrix (ECM) glycoprotein has a rather restricted tissue distribution, but until now factors that inhibit its expression have not been identified. Glucocorticoids are known to be beneficial for establishment of myelopoiesis in long-term bone marrow cultures. Tenascin was found to be expressed in the bone marrow, and glucocorticoids were found to affect bone marrow tenascin expression. Both tenascin mRNAs and the mRNA of another ECM protein, laminin B1 chain, were drastically downregulated by glucocorticoids during initiation of bone marrow cultures. However, in already established long-term cultures glucocorticoids did not affect laminin B1 chain mRNA levels although tenascin mRNAs continued to be downregulated. Studies with a stromal cell line (MC3T3-G2/PA6) and fibroblasts (3T3) suggested that glucocorticoids act directly on the stromal cells that produce tenascin. In 3T3 cells this downregulation occurred within 12 h of glucocorticoid-treatment, suggesting that glucocorticoids acted through *cis* regulatory elements of the tenascin gene. We suggest that glucocorticoids in part regulate hematopoiesis by modifying the ECM. Furthermore, downregulation of tenascin expression by glucocorticoids may in part explain the restricted tissue distribution of tenascin in other tissues.

TENASCINS are large extracellular matrix (ECM)[1] glycoproteins arranged as polymers of up to six chains. Each chain contains a series of EGF-like domains at the amino-terminal end followed by a series of fibronectin type III domains, and a fibrinogen type domain at the carboxy-terminal end (Spring et al., 1989; Jones et al., 1989; Nörenberg et al., 1992). The first described tenascin was recently named tenascin-C (Bristow et al., 1993), but it will here be referred to as tenascin. It has received much attention because of its expression in healing wounds and in the stroma close to epithelial cells during embryogenesis and carcinogenesis (Chiquet-Ehrismann et al., 1986; Ekblom and Aufderheide, 1989). It is also found in the cerebellum, developing cartilage, and some other tissues (Koukoulis et al., 1991; Natali et al., 1991). However, its tissue distribution is more restricted than several other ECM proteins such as fibronectin and the major laminins. Transforming growth factor β stimulates expression of many ECM components including tenascin (Pearson et al., 1988; Massagué, 1990) but the limited expression of tenascin in vivo suggests mechanisms that downregulate tenascin expression in the body. Indeed, fibroblasts which in vivo produce very little tenascin rapidly begin to produce tenascin when cultured in vitro.

Yet, physiological inhibitors of tenascin expression have not been identified. In experiments with cells treated with glucocorticoids we noted that tenascin expression might be affected by the hormone. To learn more about regulation of tenascin synthesis, we therefore studied tenascin expression in long-term bone marrow cultures, an organ culture system where glucocorticoids are required for differentiation.

Long term bone marrow cultures, which consist of a heterogenous population of stromal and hematopoietic cells can be used as in vitro models for hematopoiesis (Dexter et al., 1977; Whitlock and Witte, 1982; Kincade et al., 1989). Glucocorticoids have been suggested to influence the stromal layer (Greenberger, 1978; Suda and Dexter, 1981; Eliason, 1984), but it is not clear at the molecular level how glucocorticoids act in these cultures. Glucocorticoids have been reported to downregulate collagen expression in adult but not embryonic fibroblasts (Russell et al., 1989) and to upregulate fibronectin expression (Oliver et al., 1983). However, the effect of glucocorticoids on the ECM of bone marrow cells has not been studied in any detail. Glucocorticoids were recently shown to cause subtle changes in the sugar composition of bone marrow stromal proteoglycans (Siczkowski et al., 1992) but other more profound effects may occur. We here report that bone marrow contains both major splice variants of tenascin and that they continue to be expressed in long-term bone marrow cultures. Laminin B chains and nidogen were also found in the native bone mar-

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1. *Abbreviation used in this paper*: ECM, extracellular matrix.

row and in the cultures, suggesting that the marrow cultures can be used to study physiologically relevant cell–matrix interactions. We found that glucocorticoids downregulated tenascin and laminin B1 chain expression during initiation of long-term bone marrow cultures. Interestingly, only tenascin expression was affected in established long-term bone marrow cultures.

There is previous evidence that the ECM could participate in the regulation of hematopoiesis. Stromal cells as well as stem cells and lymphoid and myeloid progenitors express several known cell adhesion molecules (Hemler, 1988; Miyake et al., 1990; Teixidó et al., 1992). Stromal cells also synthesize and deposit a large number of known ECM components (Bentley and Foidart, 1980; Del Rosso et al., 1981; Zuckerman et al., 1985; Long and Dixit, 1990). Inhibitors of collagen deposition decrease cell proliferation (Zuckerman et al., 1985), while stimulation of glycosaminoglycan synthesis enhances hematopoiesis (Spooncer et al., 1983). Of the defined components, fibronectin, collagen I, and thrombospondin have been shown to be adhesive for hematopoietic cells (Lanotte et al., 1981; Giancotti et al., 1986; Weinstein et al., 1989; Verfaillie et al., 1991), but the adhesion capacity of the cells depends on the lineage and stage of differentiation (Coulombel et al., 1988; Koenigsmann et al., 1992; Long and Dixit, 1990; Williams et al., 1991). The binding to fibronectin induces differentiation of immunoglobulin-secreting cells of the marrow (Roldán et al., 1992) and murine erythroleukemia cells (Patel and Lodish, 1987), and stimulates proliferation of erythroid cells (Weinstein et al., 1989). Granulocyte progenitors also adhere to fibronectin, although to a lesser degree than erythroid cells (Tsai et al., 1987; Koenigsmann et al., 1992; Roldán et al., 1992). Tenascin has been shown to modulate the binding of monocytes and other cells to fibronectin (Chiquet-Ehrismann et al., 1988, 1991; Erickson and Bourdon, 1989). It is thus likely that the modification of the ECM composition by glucocorticoid hormones is important for hematopoiesis.

Materials and Methods

Animals and Cells

C57 black mice (6–14 wk old) were used for long-term bone marrow cultures. For immunofluorescence and immunoblotting bone marrow from DBA/1, NMRI, or C57bl × National Medical Research Institute (NMRI) mice were used.

Long-Term Bone Marrow Cultures

Bone marrow cultures of myeloid cells were initiated and maintained according to Dexter et al. (1977). 1.9–6 × 10^6 cells/ml from femurs were cultured in Nunclon tissue culture flasks. The culture medium consisted of Fischer's medium (GIBCO BRL, Gaithersburg, MD), 20% horse serum (GIBCO BRL) and 10^{-6} M hydrocortisone sodium succinate (Solu-Cortef, Upjohn, Kalamazoo, MI). After 1 wk of culture two thirds of the initial volume of fresh medium was added. Half of the medium and non-adherent cells were thereafter replaced by fresh medium weekly. The cultures were incubated in 5% CO_2 at 33°C. In some cultures hydrocortisone was omitted or replaced by 10^{-5} or 10^{-7} M dexamethasone (Sigma Chemical Co., St. Louis, MO) at onset of the cultures or 3 wk after culture in the presence of hydrocortisone. The MC3T3-G2/PA6 preadipocyte cells (Kodama et al., 1982) and 3T3 or 3T3-L1 fibroblast cells (Green and Meuth, 1974) were grown in DME (GIBCO BRL), 10% FCS at 37°C in 5% CO_2. For morphological analyses, cytocentrifuge preparations of non-adherent and trypsin-treated adherent cells were stained with May-Grünwald-Giemsa.

Antisera and Antibodies

Tenascin was detected in immunoblots and immunofluorescence with a rat monoclonal IgG_1 antibody MTn 12 reacting with both major mouse tenascin polypeptides of 260 and 210 kD. For immunoprecipitations, a mixture of the mAbs MTn 12 and MTn 15 (IgG 2a) was used. The mAbs were purified by Bakerbond Abx ion exchange chromatography using fast protein liquid chromatography (FPLC) as described (Aufderheide and Ekblom, 1988). Laminin was detected with a rabbit antiserum against mouse Engelbreth-Holm-Swarm (EHS) tumor. This antiserum reacts with A, B1, and B2 chains of laminin (Klein et al., 1988, 1990). The A chain of laminin was detected with mAb 201 as a purified IgG (Sorokin et al., 1992). An affinity-purified rabbit antiserum was used to detect nidogen (Paulsson et al., 1987; Mann et al., 1988). S-chain of laminin was detected with rat mAb D5 kindly provided by Dr. J. Sanes (Washington University, St. Louis, Missouri). Fibronectin was detected with a polyclonal antiserum specific for mouse fibronectin (a gift from Dr. K. Rubin, Biomedical Center, Uppsala University, Uppsala, Sweden). Fluorescein anti-rat and rhodamine conjugated anti-rabbit antisera were from Dianova GmbH (Hamburg, Germany) and Dako Corp. (Carpinteria, CA). The biotinylated anti-rabbit and anti-rat antibodies and streptavidin-fluorescein were from Amersham International (Amersham, UK).

Immunohistochemical Analysis

Femurs from adult mice were frozen in liquid nitrogen, and the bone marrow was then dissected on dry ice under a dissecting microscope. The material was overlaid on dry ice with Tissue Tek (Miles, Naperville, IL) and immediately thereafter frozen in liquid nitrogen. From newborn mice the femurs were frozen in Tissue Tek together with the surrounding tissues. Cryostat sections (5–7 µm) were fixed with −20°C methanol for 5 min, washed with PBS and incubated with 5% horse serum in PBS for 5 min. Monoclonal anti-tenascin antibody was used at 50 µg/ml, monoclonal anti-laminin A antibody at 8 µg/ml, anti-laminin antiserum was used diluted 1:1,000, monoclonal anti-laminin s-chain antibody 1:100, and anti-nidogen antiserum 1:100. The secondary antibodies, fluorescein and rhodamine-conjugated anti-rat and anti-rabbit antisera were used diluted 1:200. In control stainings the primary antibody was omitted or nonimmune rabbit serum (dilution 1:1,000) was used instead of the primary antiserum. Immunofluorescent stainings of long-term cultures grown on coverslips were performed after fixation with methanol. Immunolocalization studies were performed with cultures initiated and maintained in the presence of hydrocortisone.

Immunoblotting

For immunoblotting the adult mouse bone marrow was isolated frozen after immersion of the femur in liquid nitrogen. From bone marrow cultures adherent cells were scraped off in the buffer. Tissues were homogenized by sonication in TBS, pH 7.4, containing 10 mM EDTA and protease inhibitors (Paulsson et al., 1987). After sonication the homogenates were kept on ice for 1 h, centrifuged, and the protein content of the supernatants was determined with the Bio-Rad Laboratories (Richmond, CA) protein assay. As controls, extract from EHS tumor (Klein et al., 1988) was used. Before electrophoresis the samples were boiled for 5 min in Laemmli buffer containing DTT. Proteins were separated on a 4–15% Mini-Protean II gradient gel (Bio-Rad Laboratories). Rainbow™ molecular weight markers (Amersham) were run in parallel to estimate the apparent molecular weights. As blocking solution 3% BSA (globulin free; Sigma Chemical Co.) and 0.05% Tween-20 was used. Purified anti-tenascin antibodies were used at 50 µg/ml, anti-laminin antiserum at dilutions 1:1,000–300, and anti-nidogen antiserum at 1:75. The biotinylated anti-rabbit or anti-rat antibodies were used at 1:200 dilution and streptavidin-biotinylated HRP complex (Amersham) at 1:400 dilution in PBS. The color reaction was performed with 4-chloro-1-naphthol and H_2O_2.

Immunoprecipitation

Monolayers of MC33T3-G2/PA6 fibroblasts, confluent to ~75%, were cultured either untreated or treated with 10^{-5} M and 10^{-7} M dexamethasone for 48 h. Cells were then washed with PBS, and incubated with methionine- and cysteine-free labeling media containing [^{35}S]methionine-cysteine for 24 h in the presence or absence of the same concentrations of dexamethasone. Control plates received ethanol instead of ethanol+dexamethasone during the labeling period. After labeling the media were removed and
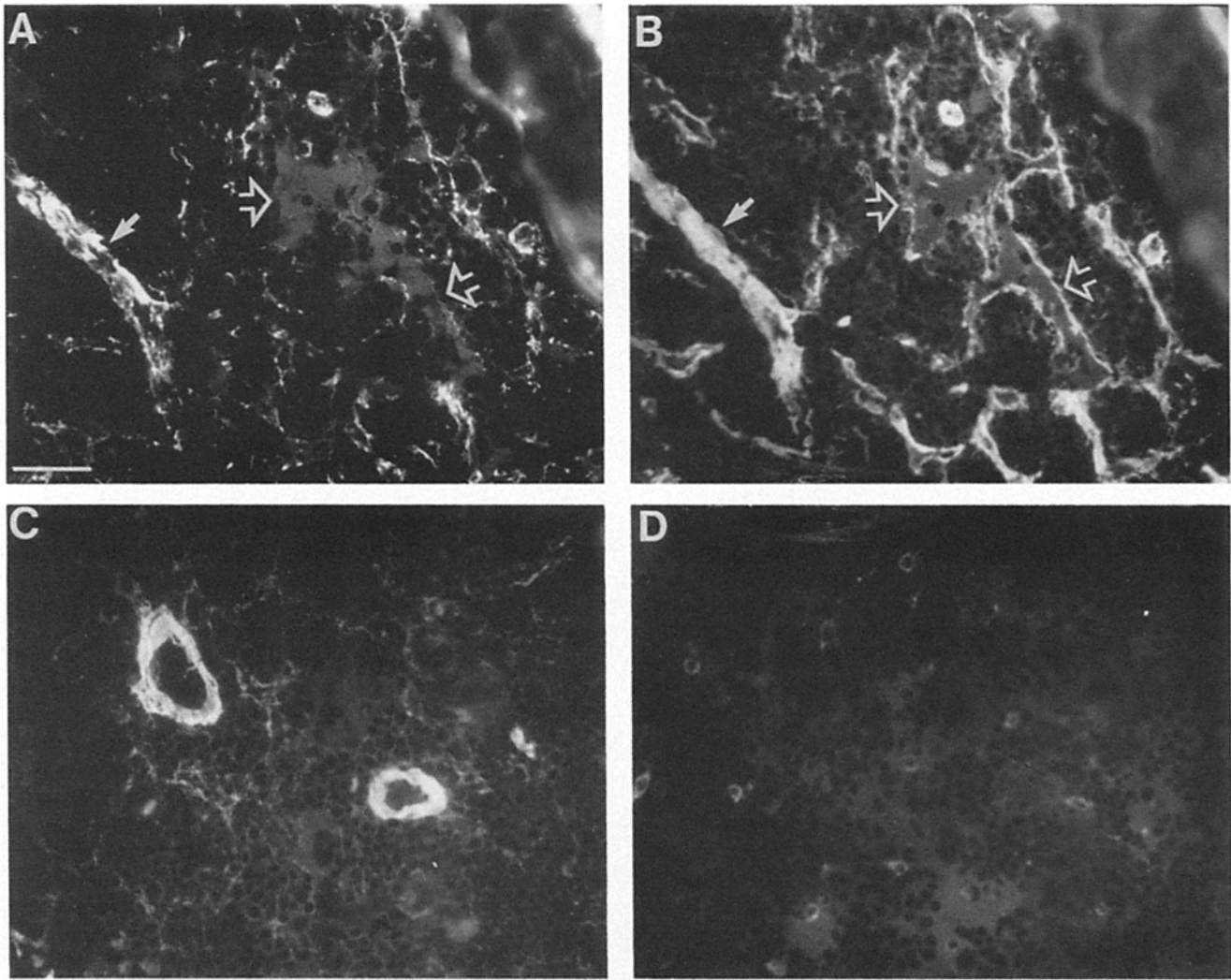
Figure 1. Tenascin, laminin, and nidogen in the bone marrow of newborn mouse. Immunofluorescence staining with anti-tenascin antibody mTn 12 shows tenascin as thin fibers among the bone marrow cells (*A*). Double staining of the same section with the anti-laminin antiserum detecting both A and B chains (*B*) shows bright staining in the muscle layer of the arteriole (*closed arrow*) and in the wall of the sinusoid (*open arrows*). In addition, there are thin fibers with laminin around cells within the bone marrow. Staining with anti-nidogen antiserum shows reaction in the muscle layer of the arteriole and in the wall of the sinusoid (*C*), whereas staining of the same section with monoclonal anti-laminin A chain shows no staining above background (*D*). Bar, 100 μm.

1 mM PMSF and 70% ammonium sulfate were added. After 3 h at 4°C, the media were centrifuged at 3,000 rpm for 20 min. Pellets were resuspended in 0.5 ml TBS and dialyzed against TBS. Samples were precleared by addition of 25 μg/ml normal rabbit IgG, 25 μg/ml rat anti-mouse IgG, protein A–Sepharose and protein G–Sepharose (Pharmacia, Uppsala, Sweden) by mixing for 2 h at 4°C. Precleared material was aliquoted into samples containing equal amounts of cpm. Samples were incubated for 2 h at 4°C with 40 μg/ml of MTn 12 and MTn 15, or with 13 μg/ml rabbit anti-rat fibronectin, or 30 μg/ml rat anti–mouse IgG. Rabbit anti-rat (25 μg/ml) and 30 μl protein A–Sepharose was added to bind MTn 12 (IgG1) and rat anti–mouse IgG-containing samples. Protein G–Sepharose (30 μl) was added to bind MTn 15 (IgG 2a)-containing samples. Protein A–Sepharose was added to rabbit anti-rat fibronectin-containing samples. Incubation with protein beads was for 1 h at 4°C. After spinning, pellets were washed 3 times with RIPA (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS in TBS containing 0.6 mM MgSO₄ and 1 mm CaCl₂), twice with 0.5% Triton X-100, 0.5 M NaCl, and once with 0.25% Triton X-100, 0.25 M NaCl. Pellets were treated with sample denaturing buffer, TBS and DTT and boiled for 5 min. 50 μl of sample were loaded per well on 5% PAGE-SDS gels. Gels were fixed, treated with fluorography enhancer, dried and exposed on x-ray film.

Northern Blotting

Total RNA was isolated from 3T3 cells as described by Auffray and Rougeon (1980). RNA from other cells and tissues was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). RNA was electrophoretically separated, blotted onto Zeta-Probe membrane (Bio-Rad Laboratories), UV cross-linked and probed in Church buffer (Church and Gilbert, 1984) at 65°C. The following oligolabeled cDNA probes were used: (*a*) p-5′6 corresponding to the 5′ region of mouse tenascin; (*b*) M20/1 corresponding to the middle region of mouse tenascin (Weller et al., 1991); (*c*) the Sph–HindIII fragment of cDNA clone for mouse laminin B1 chain (Oberbäumer, 1986); (*d*) a clone for mouse β1-integrin subunit detecting two polyadenylation variants of the mRNA; (*e*) a clone for mouse β-actin (Minty et al., 1983); (*f*) and a clone for human G3PDH (Clontech, Palo Alto, CA). Upon hybridization the blot with 3T3 RNA was washed twice for 30 min at 65°C in a solution containing 0.1 × SSC and 1% SDS. The other blots were washed twice with 0.02 M Na₂HPO₄, 5% SDS and twice with 0.02 M Na₂HPO₄, 1% SDS for 60 min at 65°C (Sambrook et al., 1989). The blots were exposed at −70°C on a Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY) for 1–14 days. Quantitation of signal intensities were performed by densitometric scanning
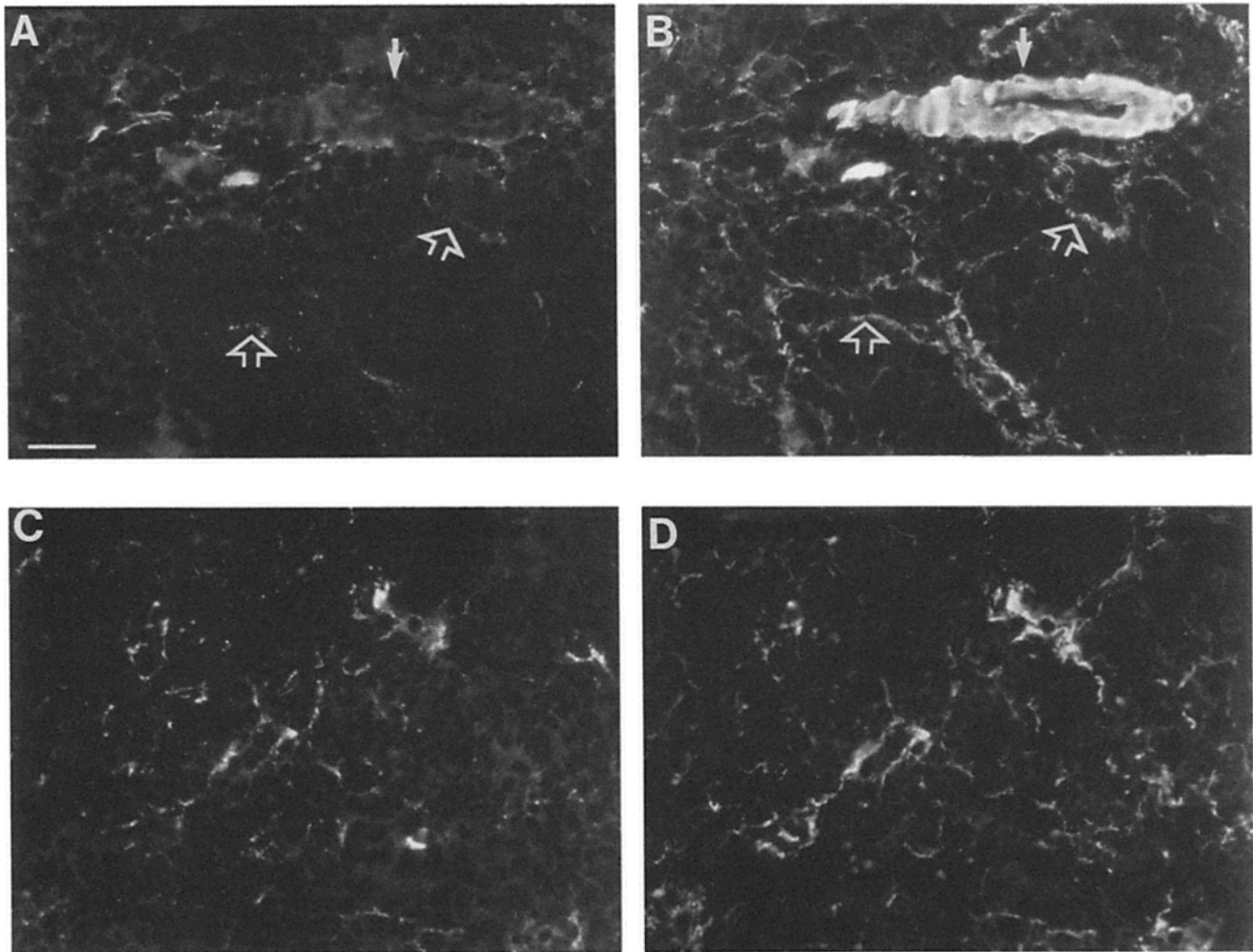
Figure 2. Laminin and tenascin in the adult mouse bone marrow. Sections of the central bone marrow (*A* and *B*) and more peripheral areas (*C* and *D*) were double stained for tenascin (*A* and *C*), and laminin A and B chains (*B* and *D*). Open arrows show sinusoidal walls, and closed arrows show the blood vessels. Bar, 50 μm.

of Northern blots using an LKB laser densitometer. Values were normalized to G3PDH signal intensities.

Results

Localization of Laminin and Tenascin in the Bone Marrow and in Long-Term Bone Marrow Cultures

In bone marrow of newborn mice tenascin was expressed as thin fibers in the intersinusoidal spaces among the bone marrow cells. Tenascin was also expressed in the walls of arteries but not in the sinusoidal linings (Fig. 1 *A*). The distribution of laminin was much more widespread then tenascin. Laminin was expressed in the walls of the arteries and veins and also in the sinusoidal walls (Fig. 1 *B*). Thin strands were also seen in extravascular areas close to bone marrow cells, as reported previously for rat (Hamilton and Campbell, 1991). The staining pattern for nidogen was identical to the laminin B chain polypeptides in newborn (Fig. 1 *C*) and adult (not shown) mouse bone marrow. A monoclonal antibody 201

against laminin A chain did not show any reaction above background (Fig. 1 *D*). By immunoblotting or immunofluorescence we failed to detect the s-chain of laminin in mouse or rat bone marrow (data not shown).

In the adult bone marrow staining for tenascin remained positive, but was weaker in the intersinusoidal spaces than in newborn bone marrow. Tenascin expression was particularly weak in the central area of the adult bone marrow, also in the arteriolar walls (Fig. 2 *A*), but more expression was seen in the subcortical areas (Fig. 2 *C*). Laminin chains remained well expressed in the adult bone marrow both in the blood vessel walls (Fig. 2 *B*) and in the sinusoidal walls (Fig. 2, *B* and *D*). Tenascin and laminin continued to be well expressed in the long-term in vitro cultures of adult bone marrow. In the cultures tenascin was seen as fibers between the stromal cells (Fig. 3 *A*), whereas laminin was seen as intracytoplasmic perinuclear deposits in some large stromal cells, but also in the extracellular matrix (Fig. 3 *B*). In the ECM of the bone marrow cultures no extensive codistribution of tenascin and laminin was noted.
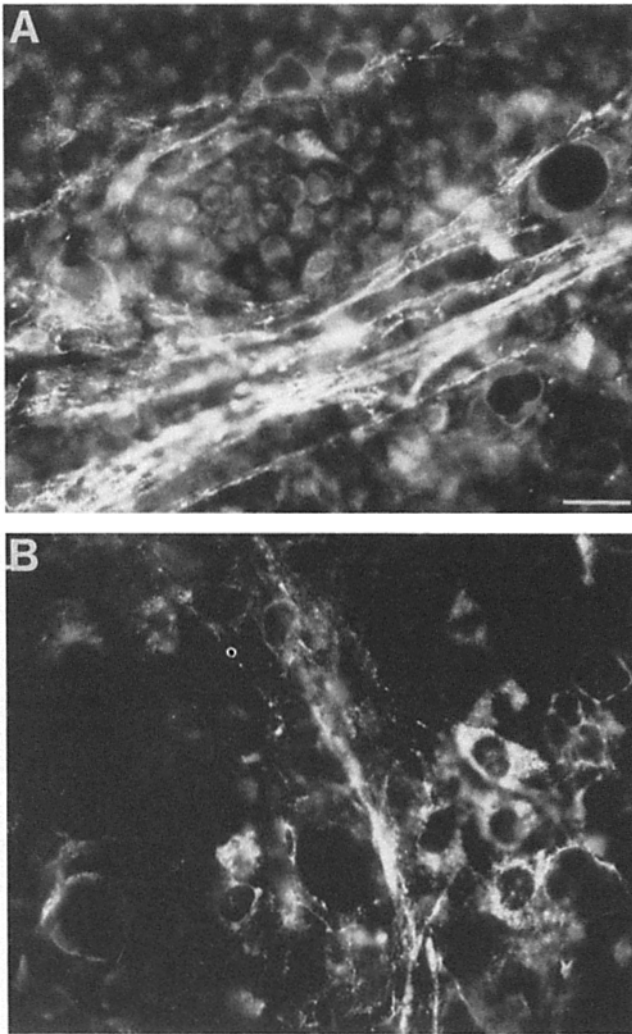
Figure 3. Localization of tenascin and laminin in the adherent layer of long-term bone marrow culture. Cells grown on a coverslip were stained with anti-tenascin antibody MTn12 (A). Tenascin is seen extracellularly arranged in large fibrils. Immunofluorescence staining with the anti-laminin antiserum shows some extracellular deposition of laminin, but laminin is predominantly found within the stromal cells perinuclearly (B). Bar, 20 μm.
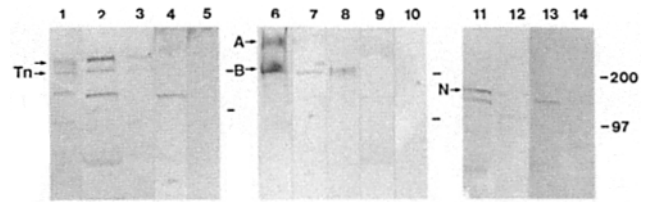

Figure 4. Polypeptide chain composition of laminin and tenascin in the bone marrow, in cultured marrow and in cultured cells. Immunoblotting was performed with monoclonal anti-tenascin antibody MTn 12 (lanes 1–3) or with second antibody alone (lanes 4 and 5), polyclonal anti-laminin (lanes 6–8) or nonimmune rabbit serum (lanes 9 and 10), and anti-nidogen antiserum (lanes 11 and 12) or nonimmune rabbit serum (lanes 13 and 14). Lysates blotted were from cell line MC3T3-G2/PA6 (lane 1), adherent cells from long-term bone marrow cultures (lanes 2, 4, 7, 9, 11, and 13), adult mouse bone marrow (lanes 3, 5, 8, 10, 12, and 14), and EHS-extract (lane 6). The second antibodies used alone frequently reacted strongly with bands of ~130 kD in the extracts from the cultured bone marrow (lane 4), but not from the native bone marrow (lane 5) which explains the extra bands in lanes 1 and 2. Two tenascin polypeptides (arrows) are visible in the cell line MC3T3-G2/PA6 (lane 1), in bone marrow cultures (lane 2), and in bone marrow (lane 3). Although the anti-laminin antiserum reacts both with the 400-kD laminin A chain and the 200-kD B chains (arrows) in the EHS extract (lane 6), only B chains were detected with the same antiserum in extracts from bone marrow cultures (lane 7) or adult mouse bone marrow (lane 8). The anti-nidogen antiserum detects a polypeptide of 150 kD (arrow) in the marrow cultures (lane 11) and in the bone marrow (lane 12). In control immunoblots of bone marrow (lanes 5 and 10) and bone marrow cultures (lanes 4, 9, and 12) with the secondary anti-rat (lanes 4 and 5) and anti-rabbit (lanes 9, 10, 13 and 14) antiserum the primary antibodies were omitted. Right margin indicates $M_r \times 10^{-3}$. Gels were run under reducing conditions. Tn, tenascin; A, laminin A chain; B, laminin B chains, and N, nidogen.

Chain Composition of Tenascin and Laminin in the Bone Marrow

Immunoblotting revealed the presence of both the 260- and 210-kD tenascin polypeptides in cultures of the established preadipocyte-stroma cell line (Fig. 4, lane 1), in the long-term cultures (Fig. 4, lane 2) and in the native bone marrow (Fig. 4, lane 3). There was a slight predominance of the larger polypeptide in all these samples. Laminin in tissues exists as several different isoforms which differ by the polypeptide chain composition. Isoforms devoid of the laminin A-chain polypeptide are particularly common both in embryonic and adult tissues, but so far there has not been any information about the subunit composition of bone marrow laminin. With an A-chain–specific mAb we failed to detect the A chain in immunofluorescence (see Fig. 1). In agreement with these findings, we detected only 200-kD B-chain polypeptides in the native bone marrow (Fig. 4, lane 8) and

in the cultured stromal cells (Fig. 4, lane 7) with an antiserum that detects both A and B chains of laminin from the EHS tumor (Fig. 4, lane 6). Immunoblotting for nidogen, a 160-kD protein associated with laminin (Timpl, 1989), showed a 160-kD band from the culture cells and the native bone marrow. Another weak 100-kD nidogen band was seen in bone marrow lysates, probably a degradation product of nidogen due to endogenous or exogenous proteases (Fig. 4, lanes 11 and 12). The results and the immunofluorescence strongly suggest that the cultured stromal bone marrow cells continue to produce the same isoforms of laminin and tenascin as the native adult bone marrow.

Downregulation of Tenascin Expression in Bone Marrow Cultures by Glucocorticoids

We first studied whether glucocorticoids affected tenascin or laminin expression in bone marrow cultures during the initial formation of the stromal cell layer. When glucocorticoids were present from the onset of the bone marrow cultures, a nearly confluent adherent cell layer, a stroma, formed after 3 wk. In contrast, the stroma was less well developed when cultures were initiated without glucocorticoid supplementation, as reported (Suda and Dexter, 1981). A transient increase in the number of nonadherent cells occurred on the
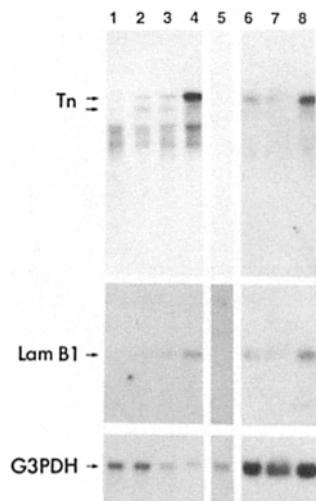
1 2 3 4 5 6 7 8

Tn

Lam B1 →

G3PDH →

Figure 5. Glucocorticoids downregulate tenascin and laminin B1 and mRNAs during initiation of long-term bone marrow cultures. Northern blotting of tenascin and laminin B1 mRNA in long term bone marrow cultures (lanes *1–4*) and in a stromal cell line MC3T3-G2/PA6 (lanes *6–8*). Lanes *1–4* were loaded with 7 μg, lane *5* with 10 μg, and lanes *6–8* with 12 μg of total RNA, and Northern blotting was performed with oligolabelled tenascin cDNA TN-20/1 (*Tn*) and a cDNA clone for mouse laminin B1. Loading in each gel was monitored by probing with a cDNA detecting G3PDH mRNA. Total RNA was isolated from the adherent cell layer of bone marrow cultures initiated in the presence of 10^{-6} M hydrocortisone (lane *1*), 10^{-5} M dexamethasone (lane *2*), 10^{-7} dexamethasone (lane *3*), or absence of glucocorticoids (lane *4*), and from the non-adherent cells grown in the presence of 10^{-6} M hydrocortisone (lane *5*). Similar Northern blots were performed with the MC3T3-G2/PA6 cell line grown in the presence of 10^{-7} M dexamethasone (lane *6*), 10^{-5} M dexamethasone (lane *7*), or without glucocorticoids (lane *8*). *Tn*, tenascin; *lamB1*, laminin B1 chain; and *G3PDH*, glycerol 3-phosphate dehydrogenase.

second week in cultures lacking glucocorticoids. This correlated with tenascin mRNA expression; glucocorticoids drastically diminished the steady state levels of tenascin mRNA in the adherent layer. Laminin B1 mRNA expression was also diminished to the same degree (Fig. 5). Non-adherent cells from the cultures grown in the presence of hydrocortisone did not express any detectable tenascin mRNA or laminin B1 (Fig. 5). Densitometric analysis of RNA signal intensity suggested that glucocorticoids de-

creased expression of tenascin and laminin mRNA in the adherent layer by ∼70–90% (Fig. 6 *B*).

We next studied whether omission of glucocorticoids could affect enascin or laminin expression once the adherent stromal cell layer had been established. Cultures were first grown for 3 wk in the presence of 10^{-6} M hydrocortisone, and were then shifted to different culture conditions containing either no steroids, 10^{-6} M hydrocortisone or 10^{-5} or 10^{-7} M dexamethasone. During the culture period there were no obvious qualitative or quantitative morphological differences between the cultures in the nonadherent cells. Omission of glucocorticoids led to increased levels of tenascin mRNA, whereas they remained low in cultures supplemented with hydrocortisone or dexamethasone. In contrast, laminin B1 mRNA steady-state levels were not in any way affected by differences in glucocorticoid concentration in these cultures (Fig. 6 *C*).

Downregulation of Tenascin Expression by Glucocorticoids in Established Cell Lines

To study whether the inhibition of tenascin expression by the glucocorticoids was due to a direct effect on stromal cells or mediated through another cell type, two established cell lines were used. In both the preadipocyte cell line MC3T3-G2/PA6 (Fig. 5) and in 3T3 cells (Fig. 6 *A*) glucocorticoids drastically downregulated the steady-state levels of tenascin mRNA. In a time course study performed with 3T3 cells the response reached a maximum at the mRNA level (∼90% inhibition) already within 12 h of glucocorticoid treatment, and persisted during the whole 6-d culture period (Fig. 7). In contrast, no downregulation of the expression of β1-integrin mRNA could be seen, either by a 1-wk treatment with 100 nM dexamethasone or by a short treatment with a high dose of 10 μM for 24 h (Fig. 7). Immunoprecipitation of metabolically labeled cells confirmed that tenascin polypeptide synthesis was reduced by dexamethasone treatment. In fibroblasts (MC3T3-G2/PA6) analyzed on the second day of culture, dexamethasone strongly reduced synthesis of the 260-kD tenascin polypeptide variant, but only slightly reduced the synthesis of the 210-kD polypeptide (Fig. 8). No reduction of synthesis of fibronectin was noted (Fig. 8).
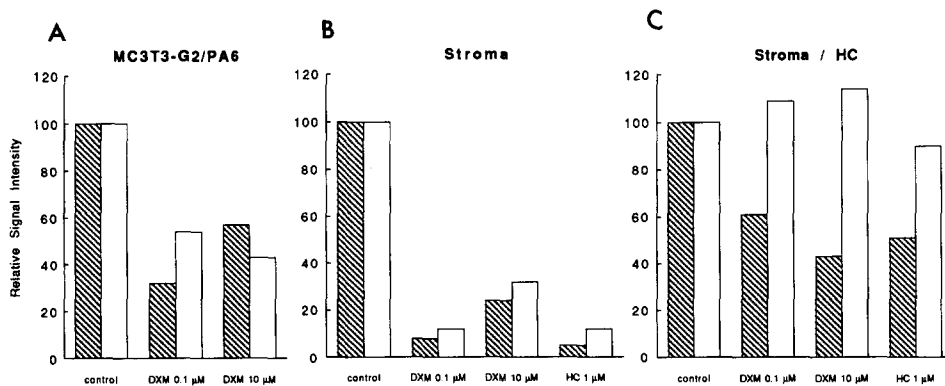

Figure 6. Differentiation-dependent regulation of the ECM by glucocorticoids. Densitometric analysis of Northern blotting for tenascin (▨) and laminin (□) were normalized to G3PDH signal intensities. MC3T3-G2/PA6 cells were cultured for one week in the presence or absence of dexamethasone (*DXM*) (*A*). Bone marrow stromal cells (*stroma*) were initiated in the presence of dexamethasone, hydrocortisone (*HC*) or without hormones and RNA levels for tenascin and laminin B1 were analyzed (*B*). In another set of experiments the stroma was first allowed to develop well in the presence of hydrocortisone for 3 wk (*stroma/HC*). Cultures were then exposed to different concentrations of glucocorticoids or cultured without hormones, and analyzed for tenascin and laminin B1 expression after 1 wk (*C*). Numbers are percent of signal intensities of cultures grown without glucocorticoids (*control*).
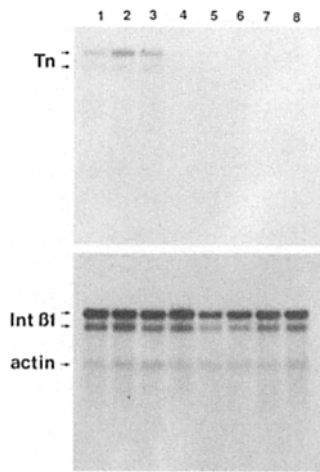
Figure 7. Time course study of the effect of glucocorticoids on steady-state levels of tenascin mRNA. 3T3 cells were grown without glucocorticoids (lane *1*), in the presence of 100 nM dexamethasone for 4 h (lane *3*), 12 h (lane *4*), 24 h (lane *5*), 4 d (lane *6*), and 6 d (lane *7*), or in the presence of 10 μM dexamethasone for 24 h (lane *8*). A control blot of cells grown for 24 h in the presence of ethanol only, used to solubilize dexamethasone is provided to show that ethanol does not influence tenascin expression (lane 2). 10 μg of total RNA from each time point was probed with oligolabelled cDNA p5'-6 detecting tenascin mRNA and cDNA detecting the two polyadenylation variants of the mouse β1-integrin subunit. Blotting with a cDNA for β actin shows that an equal amount of RNA was loaded. At higher exposure levels not shown here, some expression of tenascin was seen in all lanes. *Tn*, tenascin; *Intβ*, integrin β1 subunit.

Discussion

Glucocorticoids are known to be important for myelopoiesis in vitro but the molecular mechanisms of the effect are not clear (Dexter and Spooncer, 1987; Dorshkind, 1990). Our current study raises the possibility that glucocorticoids affect hematopoiesis by modifying the ECM. During initiation of long-term bone marrow cultures glucocorticoids drastically downregulated the steady-state levels of both tenascin and laminin B1 chain mRNA, by >80%. Interestingly, no such effect could be seen on laminin mRNA once the bone marrow stroma cell layer had formed whereas tenascin mRNA levels continued to be downregulated. In bone marrow cultures many interacting cell types are present, but our findings on 3T3 fibroblastic and MC3T3-G2/PA6 preadipocyte cell lines suggest that glucocorticoids act directly on the stromal cells that produce tenascin.

By immunohistology tenascin and laminin were localized in discrete locations of the bone marrow. In the newborn mouse bone marrow tenascin was widely expressed in the intersinusoidal spaces. In adult bone marrow tenascin expression in the intersinusoidal spaces was weaker and was seen predominantly in the subcortical areas. Laminin was more widely expressed within the bone marrow stroma. Neither laminin A chain nor s-chain but only chains reacting with antibodies against B1 and B2 chains were found in the marrow. Hence bone marrow laminin resembles mesenchymal rather than epithelial laminin (Klein et al., 1990; Ekblom et al., 1990). Nevertheless, the laminin binding protein nidogen was expressed in the bone marrow with a similar distribution as laminin, suggesting that laminin A-chain polypeptide is not necessary for the laminin–nidogen assembly. Since bone marrow laminin lacks the A chain it should not bind to the laminin receptor integrin α6β1 (Deutzmann et al., 1990). The biological role of bone marrow laminin is therefore unclear. However, it could be involved in a variety of adhesive events during hematopoiesis. Anti-laminin antibodies
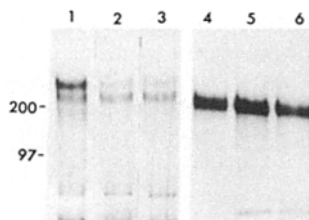

Figure 8. Reduction of tenascin polypeptide biosynthesis by dexamethasone. MC3T3-G2/PA6 cells were cultured for 48 h in the absence of dexamethasone (lanes *1* and *4*) or presence of 10^{-5} M (lanes *2* and *5*) and 10^{-7} M dexamethasone (lanes *3* and *6*). After a 48-h culture in the indicated concentrations of dexamethasone cells were metabolically labelled for 24 h by [³⁵S]methionine–cysteine in the same concentrations of dexamethasone. Immunoprecipitation was performed with mAbs MTn 12 and MTn 15 against tenascin (lanes *1–3*) and polyclonal antiserum against fibronectin (lanes *4–6*). Immunoprecipitated proteins were analyzed by SDS-PAGE under reducing conditions. Left margin indicates $M_r \times 10^{-3}$.

have recently been shown to affect lymphocyte trafficking (Kupiec-Weglinski and de Sousa, 1991), and some leukemia cells have acquired increased ability to adhere to laminin (Verfaillie et al., 1992).

The role of tenascin is not well defined, but there is some evidence that it in vitro influences cell–matrix interactions. Tenascin has been suggested to act as an anti-adhesive agent (Chiquet-Ehrismann et al., 1986; Faissner and Kruse, 1990; Murphy-Ullrich et al., 1991; Lochter et al., 1991). It binds to chondroitin sulfate proteoglycan (Chiquet and Fambrough, 1984; Hoffman et al., 1988) and to fibronectin, and it can inhibit adhesion of cells to fibronectin (Chiquet-Ehrismann et al., 1988; Rüegg et al., 1989; Lotz et al., 1989). Our findings of tenascin expression in the bone marrow stroma are in agreement with this hypothesis. Glucocorticoids downregulated tenascin mRNAs and led to decreased tenascin polypeptide content in the adherent stromal layers in long-term bone marrow cultures. In glucocorticoid-deficient cultures increased tenascin polypeptide content was associated with transient increase in non-adherent cells at 2 wk of culture and low levels of hematopoiesis thereafter. This transient increase of nonadherent cells in glucocorticoid-deficient cultures has been explained by possible toxicity of glucocorticoids to hematopoietic progenitors (Suda and Dexter, 1981). In the light of our present studies a more likely alternative is that the glucocorticoids modulate cell–matrix interactions by influencing the biosynthesis of ECM components such as tenascin. In glucocorticoid-deficient cultures the increased expression of tenascin could lead to decreased adhesion of hematopoietic cells to other ECM components and to the stroma. Analogously, it was previously suggested that tenascin might be involved in the physiological cell shedding from intestinal villi (Probstmeier et al., 1990). The availability of the in vitro model for hematopoiesis, monoclonal antibodies that affect binding of cells to mouse tenascin (Husman et al., 1992) and cDNA clones for several species that allow the production of recombinant tenascin polypeptides (Spring et al., 1989; Jones et al., 1989; Siri et al., 1991; Nies et al., 1991; Saga et al., 1991; Weller et al., 1991) should make it possible to experimentally study whether tenascin affects adhesion of hematopoietic cells.

The mechanism of glucocorticoid regulation of tenascin synthesis is unclear. Glucocorticoid hormones act by form-

ing a complex with cytoplasmic receptors which then translocate into the nucleus. In the nucleus glucocorticoids can regulate gene expression either directly by binding to glucocorticoid responsive elements (Yang-Yen et al., 1990) or indirectly by interfering with other transcription factors (Diamond et al., 1990; Yang-Yen et al., 1990; Jonat et al., 1990). In our experiments with 3T3 cells tenascin mRNA levels were reduced by glucocorticoids already between 4 and 12 h suggesting that the hormone–receptor complex acts directly on cis regulatory elements of the tenascin gene. To study the molecular basis of glucocorticoid regulation of tenascin expression we are currently characterizing the promoter region of the mouse tenascin gene.

Although glucocorticoids strongly decreased tenascin expression, the changes in adhesive cell interactions caused by glucocorticoids in the bone marrow cultures could be due to reduced or altered expression of other adhesion proteins. Based on the current data it seems that glucocorticoids do not affect ECM synthesis as broadly as transforming growth factor β (TGF-β), which stimulates or influences synthesis of a very large number of ECM proteins, integrin receptors and enzymes degrading ECM (Massagué, 1990). Integrin $\beta1$ mRNA was not affected, suggesting that glucocorticoids do not cause a simultaneous downregulation of ECM receptors. However, laminin B1 chain mRNA was downregulated by glucocorticoids in the bone marrow during the initiation of cultures. Since a number of ECM proteins or proteases degrading ECM are affected by glucocorticoids in other tissues (Russell et al., 1989; Jonat et al., 1990) at least some other ECM components may be affected in the bone marrow as well.

The data showing that glucocorticoids strongly influence the expression of ECM components may turn out to be relevant for the understanding of clinical symptoms caused by excess of corticosteroids. These syndromes include skin atrophy, impaired bone marrow differentiation, immunological deficiency leading to increased susceptibility to infections, and a reduced wound healing capacity. There are no satisfactory explanations at the molecular level for the many side effects of glucocorticoids. It is tempting to speculate that at least some of them could be related to the downregulation of ECM components such as the tenascins and laminins. It will therefore be of interest to test whether the biosynthesis of other members of the tenascin and laminin families are affected by glucocorticoids.

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