Tenascin Is a Cytoadhesive Extracellular Matrix Component of the Human Hematopoietic Microenvironment

Gerd Klein, Susanne Beck, and Claudia A. Müller

University Medical Clinic, Department of Internal Medicine II, Section for Transplantation Immunology and Immunohematology, 72076 Tübingen, Federal Republic of Germany

Abstract. Tenascin is a large extracellular matrix (ECM) glycoprotein found in restricted tissue locations in the adult organism. It is copiously synthesized in regenerative organs or regenerating tissues and by certain tumors. We have analyzed the expression of tenascin in human long term bone marrow cultures as well as in cryostat sections of native bone marrow and found it strongly expressed by the stromal cells of the microenvironment. Two different protein subunits of 280 and 220 kD were detected by immunoblotting. These two forms are derived most likely from two different mRNA splice variants of 6 and 8 kb detected by Northern blotting. The in vivo analysis of cryostat sections showed a codistribution with other ECM molecules such as fibronectin and collagen type III in the microenvironment surrounding the maturing hematopoietic cells. Using two independent cell adhesion assays tenascin could be shown to function as a cytoadhesive molecule for hematopoietic cells. These data suggest a direct involvement of tenascin in the retention of hematopoietic progenitor cells in the stroma.

THE hematopoietic microenvironment, which is known to be essential for hematopoietic stem cell proliferation and differentiation, consists of different kinds of stromal cells and an extracellular matrix (ECM)[1] (15, 40). Adhesive cell–cell and cell–matrix interactions are thought to play a critical role in the specific homing of hematopoietic progenitor cells to the bone marrow and the controlled release of mature cells into the peripheral blood stream (5, 21). Many data concerning the importance of the marrow stromal cells and their ECM for hematopoiesis are derived from studies with long term marrow cultures (LTMC), in which an adherent layer of stromal cells can support hematopoiesis in vitro for months (14, 19). In these LTMC, direct cell–cell contact between hematopoietic progenitor cells and stromal cells as well as specific interactions with components of the ECM has been shown to be necessary for hematopoietic cell maturation (3, 5, 13).

Within the ECM, heparan sulfate proteoglycans can bind hematopoietic growth factors and present them to the hematopoietic precursors (22, 34). Three other extracellular matrix components are known to be involved in hematopoietic cell attachment. Many hematopoietic cell lines bind to fibronectin, a wide-spread ECM molecule (20). The attachment of developing erythrocytes to fibronectin is devel-

opmentally regulated (32, 33). Thrombospondin, another ECM molecule with a ubiquitous distribution, mediates the adhesion of pluripotent and committed progenitor cells (27). An adhesive ECM component with a specific restriction to the bone marrow is hemonectin, which has been characterized in the rabbit (6). Binding to hemonectin has been shown to be developmentally controlled for granulocytic cells (7).

Another defined ECM protein with adhesive properties is tenascin. This molecule shows a restricted expression pattern both during development and in the adult organism (17, 30). In the mouse embryo, tenascin could be detected at sites of epithelial-mesenchymal interactions which are of utmost importance during normal organ development (2, 11, 43). Despite the restricted expression pattern reported for tenascin which implicated important functions in various morphogenetic events, it was recently shown that mutant mice lacking tenascin expression develop normally (36). This unexpected finding refutes the vital role of tenascin during embryogenesis. In the adult organism tenascin expression is mainly found in skin and gut, both highly regenerating organ systems, along basement membranes of various epithelia, as well as at sites of wound healing and in certain tumors (1, 11, 26, 28). It remains to be seen if adult tissues are also not affected by the loss of tenascin in the mutant mice.

Tenascin consists of six similar subunits linked to their amino terminal ends by disulfide bonds (18). Due to differential splicing, the length of the subunits may vary and subunit composition can be tissue-specific (23, 41). Tenascin has been shown to interact with an integrin-like receptor (4), but also seems to bind particularly to proteoglycans (24).

Please address all correspondence to Dr. Gerd Klein, University Medical Clinic, Department II, Otfried-Müller Straße 10, 72076 Tübingen, FRG.

1. *Abbreviations used in this paper:* BM, bone marrow; DIG, digoxigenin-11-UTP; ECM, extracellular matrix; LTMC, long-term marrow cultures.

In this study, the expression pattern of tenascin and its possible role in cell attachment in the human bone marrow was analyzed both ex vivo and in LTMC. Using immunofluorescence staining and Western and Northern blotting tenascin is demonstrated to be a major ECM protein of this highly reproductive tissue. Two different and independent cell adhesion assays suggest a specific adhesive function of tenascin for cell–matrix interactions between stromal cells and hematopoietic progenitor cells.

Materials and Methods

Bone Marrow Samples

Bone marrow (BM) samples taken from the sternum of nonhematologic patients undergoing thoracic surgery after informed consent were kindly provided by Professor Seboldt, University Surgery Clinic, Tübingen. These samples were mainly used for cryostat sections. For the establishment of LTMC normal BM aspirates were derived from consenting donors of BM transplants from the Bone Marrow Transplantation Center, University Medical Clinic, Tübingen.

Establishment of Long Term Bone Marrow Cultures

Mononuclear cells were obtained from BM aspirates diluted with PBS after density gradient centrifugation on a Percoll cushion (1.077 mg/ml). For immunofluorescence staining, stromal cell layers were grown from 10^6 mononuclear cells in 24 well plates, either on plastic or on glass coverslips, in 1 ml RPMI-1640 culture medium supplemented with 12.5% horse serum, 12.5% FCS and 10^{-6}M hydrocortisone (Dexter conditions). For comparison, a medium according to Whitlocke/Witte containing 5% FCS and 5×10^{-5}M mercaptoethanol was used to establish LTMC mainly supporting lymphopoiesis. For blotting procedures and the indirect cell adhesion assay, BM cells were seeded at a density of 5×10^6 mononuclear cells in 5 ml in tissue culture flasks (25 cm^2) or in 2 ml in 35-mm dishes, respectively. For these studies the medium according to Dexter was used. The media were changed completely every week.

Cell Culture

The following cell lines kindly provided by Dr. H. J. Bühring (University of Tübingen, FRG) were used for cell attachment assays: HL60 (promyelocytic), KGla (myeloblastic), and K562 (erythroleukemic cell line). All these cell lines were grown in RPMI-1640 culture medium supplemented with 10% FCS. The human melanoma cell line SK-MEL-28, which was used for tenascin purification, was obtained from American Type Culture Collection (Rockville, MD). This cell line was propagated in minimal essential medium with HBSS supplemented with nonessential amino acids, L-glutamine, sodium pyruvate, and 10% FCS. Serum was omitted from this medium when the cells were used to collect conditioned medium for purification of tenascin.

Antibodies

For the unambiguous detection of tenascin in the bone marrow, different monoclonal and polyclonal antibodies were applied. A polyclonal anti-human tenascin antiserum was purchased from Telios Pharmaceuticals (San Diego, CA). Two monoclonal antibodies against human tenascin, clone EB2 and clone DB7, were commercially available from BIOHIT OY, Helsinki, Finland. Two monoclonal antibodies against mouse tenascin, MTn1 and MTn24, which cross-react with human tenascin, were a kind gift of Dr. Peter Ekblom, University of Uppsala, Sweden. The human blood vessel specific monoclonal antibody Rb10, which reacts exclusively with endothelial cells of various tissues tested (e.g., tonsils, appendix, lymph nodes and Peyer's patches) was a generous gift of Dr. Rupert Hallmann, Max Planck group for rheumatology, Erlangen, FRG. The antiserum against EHS-tumor laminin which cross-reacts with human laminin has been described earlier (25). The other antibodies were purchased from Dianova, Hamburg, FRG (mouse anti–human β1-integrin), Telios Pharmaceuticals (rabbit anti–human fibronectin), Dunn, Asbach, FRG (goat anti–human collagen type III), and Dako, Hamburg, FRG (horseradish peroxidase-conjugated rabbit anti–mouse immunoglobulins).

Indirect Immunofluorescence

BM samples were frozen in Tissue-Tek (Miles Scientific, Naperville, IL) by liquid nitrogen. 6–8-μm slices were cut on a cryostat (Reichert and Jung, Heidelberg, FRG) and air-dried for at least 1 h. Both BM cryostat sections and stromal cells grown on coverslips were fixed for 5 min in $-20°$C methanol. The fixed specimens were washed in PBS containing 0.1% albumin, and then incubated with the first antibody for 1 h in a moist chamber. For double immunofluorescence staining, the first antibodies, either an antiserum from rabbit or from goat and a monoclonal antibody from either mouse or rat, were incubated simultaneously. After several washings, the bound antibodies were detected with a FITC-conjugated anti–mouse or anti–rat second antibody and a rhodamine-conjugated anti–rabbit or anti–goat second antibody (Dianova, Hamburg, FRG) at a dilution of 1:200. The second antibodies were also simultaneously incubated. After washing with PBS, the specimens were mounted with elvanol embedding medium and photographs were taken under a Zeiss axiophot microscope equipped with epifluorescent optics. Control stainings were performed by omitting the first antibodies and no fluorescence staining above background could be detected.

Immunoblotting

Tenascin was extracted from LTMC in a buffer containing 150 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.4, 2 mM PMSF and 0.1% aprotinin. Using a cell scraper the adherent cells were scraped off, and after sonication the homogenate was kept on ice for one hour. After centrifugation the supernatant was reduced by boiling for 5 min in Laemmli buffer containing dithiotreitol. The protein extract was separated on a 5–15% SDS-polyacrylamide gradient gel and transferred onto nitrocellulose. Blocking of the filter was performed in a solution containing 3% BSA and 0.05% Tween 20 for 3 h. The filter was then incubated with the monoclonal anti-human tenascin antibodies. Bound antibodies were detected with the peroxidase conjugated anti–mouse antibody and visualized by a color reaction with H_2O_2 and 4-chloro-1-naphtol. For molecular mass determination a colored standard protein mixture (rainbow marker, Amersham-Buchler, Braunschweig, FRG) was used.

Northern Blotting

Total RNA was extracted from pooled stromal cells which were trypsinized and then homogenized in 4 M guanidinium isothiocyanate. The homogenate was forced through a 20-gauge needle several times to shear high molecular weight DNA. The RNA was purified by ultracentrifugation through a cesium chloride cushion. Around 5 μg of total RNA were obtained from 10^6 cells. The total RNA was electrophoresed in a 1% agarose formaldehyde gel (39). Transfer of RNA to a Hybond N$^+$ nylon membrane (Amersham-Buchler, Braunschweig, FRG) was carried out by capillary blotting with 20× SSC according to the manufacturer's instructions. For hybridization, the human tenascin specific 2.2 kB cDNA probe HT-11 (8), which was a generous gift of Dr. Luciano Zardi, Genoa, Italy, was used. 50 ng of the purified cDNA HT-11 were labeled with digoxigenin-11-UTP (DIG) from Boehringer-Mannheim, FRG, according to their instructions. Prehybridization of the filter and hybridization with the DIG-labeled cDNA probe was carried out exactly according to an optimized hybridization and detection protocol for nonradioactive Northern blotting recently published by Engler-Blum et al. (16). After overnight hybridization at 68°C, the filter was washed several times at 68°C. Immunological detection of the bound DIG-labeled cDNA was achieved with anti-DIG alkaline phosphatase-conjugated Fab fragments and AMPPD purchased from Boehringer-Mannheim, FRG. After sealing the wet membrane in a hybridization bag the filter was exposed to Hyperfilm™-MP (Amersham-Buchler) at room temperature for various times. The sizes of the mRNA detected were determined using a DNA molecular weight marker (Boehringer, Mannheim, FRG).

Indirect Cell Adhesion Assay

Freshly isolated BM mononuclear cells were incubated at 37°C in a plastic culture dish. After two hours of incubation the plastic nonadherent cells were removed after gentle agitation of the dish and seeded at a density of 6×10^5 cells onto preformed confluent stromal cell layers in 35-mm dishes. The incubation with the stromal layers was done in the absence or presence of different antibodies. 10 μl of the respective antisera was added in each experiment. To evaluate if an antibody had an inhibitory effect on cell binding, the stroma nonadherent cells were removed after 2 h of incubation and assayed in semisolid 0.8% methylcellulose cultures for their CFU-

GM and BFU-E capacities. 1,000 U ml^{-1} of GM-CSF (granulocyte/macrophage colony stimulating factor from Genzyme, ICC, Ismaning, FRG; specific activity 3× 10^7 U/mg) and 50 U ml^{-1} of erythropoietin (CILAG, Sulzbach, FRG) were added to these cultures. After fourteen days of culture, formed colonies with more than fifty cells were counted.

Tenascin Purification

Human tenascin was purified from conditioned medium of the SK-MEL-28 cell line according to a recently published method of Saginati et al. (37). Briefly, confluent cell layers of SK-MEL-28 were fed with serum free medium containing 1% aprotinin to inhibit protein degradation. Conditioned media for tenascin purification were completely replaced on day 7, 14, 21, and 28. The collected and combined medium was filtered, the pH was corrected to pH 7.0, and the medium was then passed through a gelatin-Sepharose-4B column in order to remove fibronectin which is a main component shed into the medium. Unbound material was directly passed through a hydroxylapatite column equilibrated with medium. Under these conditions tenascin bound to the column. After washing, the bound material could be eluted by a sodium phosphate linear gradient of 5–300 mM. The fractions containing tenascin were determined by immunoblotting with the monoclonal anti-human tenascin antibody EB2. 6% of polyethylene glycol (PEG) was added to the combined tenascin-containing fractions to remove high molecular mass contaminants. After centrifugation tenascin was precipitated from the supernatant by increasing the PEG-concentration to 12.8%. The centrifuged pellet containing the pure tenascin preparation as judged by silver staining was dissolved in 150 mM NaCl, 20 mM Tris-HCl, and aliquots were stored at −20°C.

Cell Attachment Assay

Human fibronectin purchased from Telios (Biomol, Hamburg, FRG) and purified tenascin from the SK-MEL-28 cell line were immobilized onto plastic dishes by air-drying at room temperature. 2 μg of the proteins were used for each spot. Nonspecific binding of cells to plastic was prevented by blocking the culture dishes with a solution of 1 mg/ml HSA in RPMI-1610 medium. Cells in serum free medium were allowed to adhere to the immobilized proteins for 1 h at 37°C. Nonadherent cells were removed by gently rinsing the wells with warm PBS. Adherent cells were fixed for 20 min with 3% paraformaldehyde and stained with the May-Grünwald Giemsa dye. Specific cell attachment was evaluated under a photomicroscope.

Results

Expression of Tenascin in Long Term Bone Marrow Cultures

BM stromal cells were grown on glass coverslips for at least 3 wk before fixation and immunostaining with the anti-tenascin antibodies. The immunofluorescence staining showed a strong expression of tenascin in the adherent stromal cell layer no matter whether the cells were grown under Dexter conditions favoring myelopoiesis as seen in Fig. 1 *a* or under Whitlocke/Witte conditions favoring lymphopoiesis (data not shown). In all cultures analyzed tenascin could be found in an extracellular meshwork. Double immunofluorescence staining of tenascin with other ECM molecules did not show an exact codistribution of these molecules in the stromal cell cultures. For example, the extracellular deposition of fibronectin (Fig. 1 *b*) could be shown to be different from that of tenascin (Fig. 1 *a*).

The Western blot analysis confirmed the expression of tenascin by the adherent stromal layer. Using the combined monoclonal anti-human tenascin antibodies EB2 and DB7 a strong reaction of the high molecular mass band of 280 kD was observed in stromal cell extracts. In addition a weaker band of 220 kD could be detected in these extracts (Fig. 2, lane *a*). These two signals most probably correspond to two
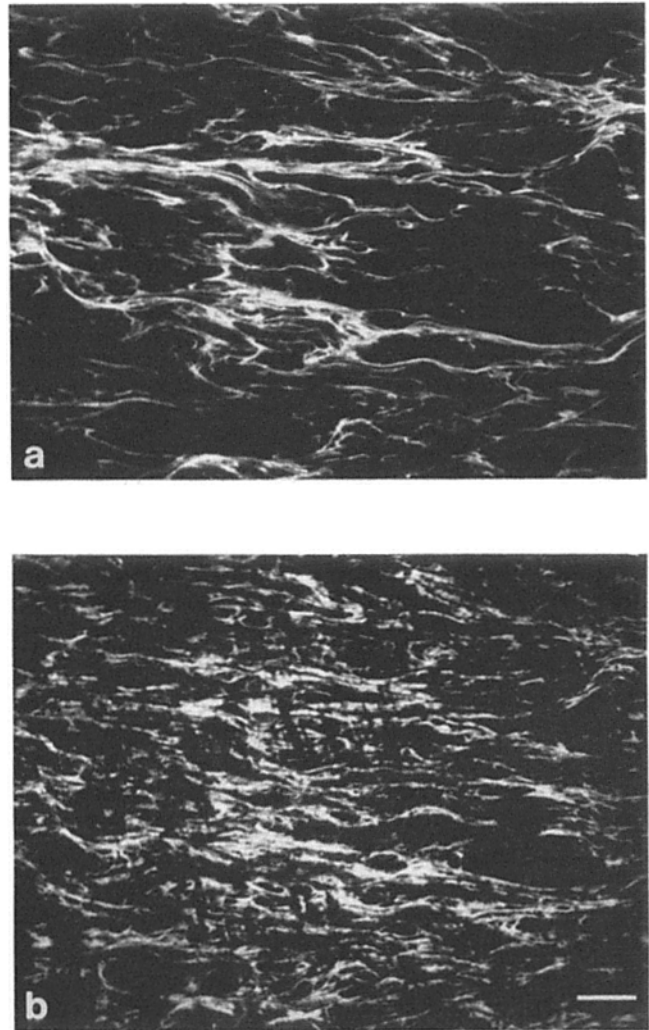

Figure 1. Immunolocalization of tenascin in LTMC. The micrographs show a double immunofluorescence staining of fixed stromal cells with an antibody against tenascin (*a*) and with an antiserum against fibronectin (*b*). The adherent stromal cells express both matrix components in an extracellular meshwork, but the density of the extracellular fibrils is less for tenascin. Bar, 50 μm.

different splice variants known for the tenascin molecule, although it cannot be excluded that the weaker 220-kD band represents a specific degradation product. To exclude that the anti-tenascin antibodies do not even slightly cross-react with fibronectin which is also strongly expressed in LTMC (Fig. 1 *b*), 1.5 μg of purified human fibronectin was immunoblotted with either the combined monoclonal antibodies (Fig. 2, lane *b*) or the polyclonal anti-tenascin antiserum (data not shown). None of these antibodies showed any reaction with purified fibronectin antigen.

Transcription of tenascin by BM stromal cells was evaluated by Northern blot analysis. Total RNA of stromal cells was probed under high stringency conditions with the human tenascin-specific cDNA clone HT-11 which reacts with an unspliced region of human tenascin. Under these conditions, both the 6-kb and the 8-kb messages of tenascin, also present in other studied tissues (44), could be detected in the stromal cell cultures (Fig. 3). Yet, the 8-kb mRNA is more strongly
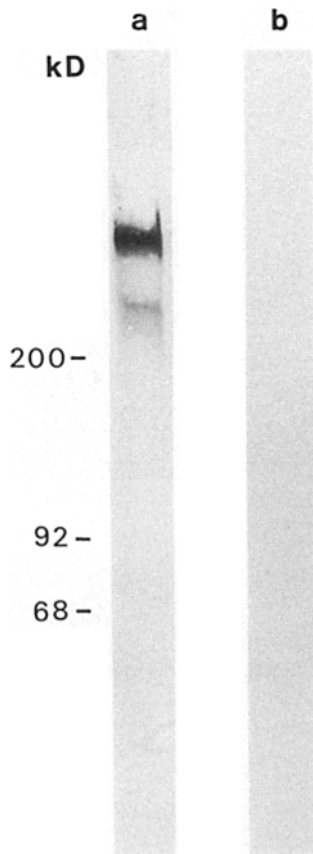
Figure 2. Immunoblot analysis of tenascin expression in LTMC. Adherent cells of an LTMC were extracted by sonication in a TBS-EDTA buffer. The cell extract (lane *a*) and 1.5 μg purified fibronectin (lane *b*) were run under reducing conditions on a 5-15% SDS-PAGE gradient gel. Using a combination of the monoclonal antibodies EB2 and DB7, a strong expression of tenascin could be found in the cell extract of LTMC (lane *a*). Two signals for tenascin could be seen, corresponding to the different splice variants. No signal at all was visible in lane *b*, thus proving that the antibodies do not cross-react with fibronectin. Relative molecular mass markers $\times 10^{-3}$ are indicated to the left of the lanes.
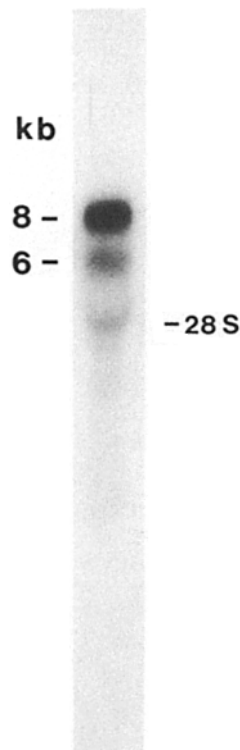

Figure 3. Northern blot analysis of tenascin in LTMC. 8.8 μg of total RNA isolated from LTMC cells were used. The filter was hybridized with the 2.2-kb cDNA clone HT-11 which detects an unspliced region of human tenascin. Two bands of 8 and 6 kb representing tenascin splice variants can be detected. The 8-kb band is more prominently expressed than the 6-kb message. Exposure time of the labeled filter was 30 min.

expressed than the 6-kb mRNA, but this result is in congruence with the obtained Western blot data showing also a stronger expression of the higher molecular weight band.

Tenascin Expression in Bone Marrow In Vivo

We analyzed the expression of tenascin in vivo on cryostat sections of BM samples and found a strong expression of this ECM protein in this tissue. Double immunofluorescence staining showed a codistribution of tenascin with fibronectin (Fig. 4, *a* and *b*) and with collagen type III (Fig. 4, *c* and *d*). This staining pattern of tenascin in the ECM surrounding the hematopoietic cells could be detected with the different specific polyclonal and monoclonal antibodies used (see Materials and Methods), which were shown not to cross-react with fibronectin. A differential expression pattern was seen on BM cryostat sections for tenascin and the β1 chain of integrins (Fig. 4, *e* and *f*). Tenascin could be detected at sites where β1 integrin was not present. This finding does not exclude that a member of the β1-integrin family can still serve as a cellular receptor for tenascin in the human BM, but there might be other tenascin receptors as well.

In addition to its localization in the extracellular space of the maturing hematopoietic cells, tenascin was also found in close association with blood vessels of the BM. In longitudinal and in cross sections of blood vessels, tenascin could be clearly localized within the walls of the arterial blood vessels (Fig. 5, *a* and *b*).

Inhibition of Progenitor Cell Adhesion to Stromal Cells by Anti-Tenascin Antibodies

To analyze adhesive properties of tenascin within the BM,

the polyclonal antiserum against tenascin was used to inhibit cell binding between hematopoietic cells and a preformed adherent stromal layer. Freshly isolated BM cells were layered onto a preformed stroma in the presence or absence of the antiserum. After an incubation time of 2 h, the non-adhering cells were transferred into semisolid colony assays for 14 d. In four independent experiments (Table I), we consistently found a significantly higher number of colonies in those cultures which were incubated with the anti-tenascin antiserum as compared to the cultures without antibody treatment, thus providing evidence that the antiserum had an inhibitory effect on cell binding of committed progenitor cells. GM-CSF and erythropoietin were added to the colony assays as growth factors, but only CFU-GM could be detected at 14 d. This suggests that the anti-tenascin antiserum did not interfere with BFU-E binding to stroma. Because of the large known donor–donor variations in progenitor cell numbers of BM cells, the total number of committed precursor cells without incubation on a stromal layer was determined in four individual experiments and the percentage of formed colonies after incubation on stroma was calculated. Such a calculation allows the specific inhibition of progenitor cell binding by the anti-tenascin antiserum which was found in all experiments conducted to be compared. The difference between the cultures grown with anti-tenascin antiserum and without antibody was found to be 12.7% ± 1.9% ($n = 4$). In control experiments two different antibodies were used. The incubation with an antiserum against laminin, an ECM component also expressed by stromal cells, had no influence on progenitor cell binding (Table I, 1.1) thus showing that the presence of an antibody alone has no inhibitory effect. In contrast, with an antiserum against fibronectin which is also strongly expressed by the stromal cells, we observed an inhibition of 14.2% of progenitor cell binding (Table I, 4.1). This inhibition is comparable to that found with the anti-tenascin
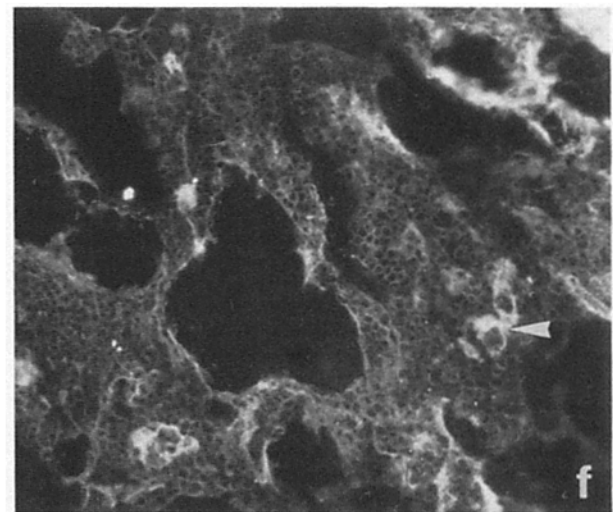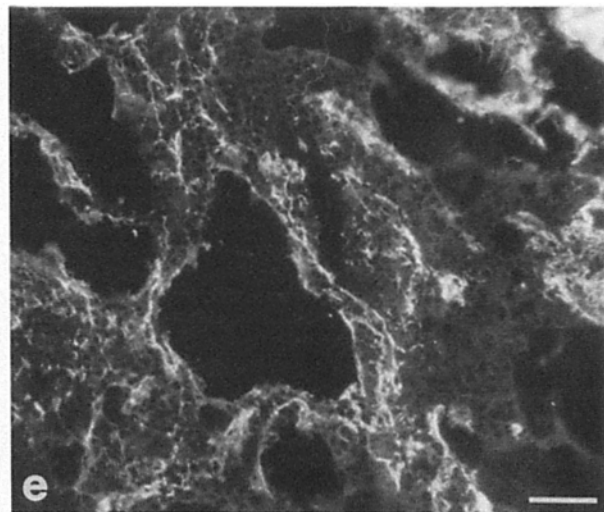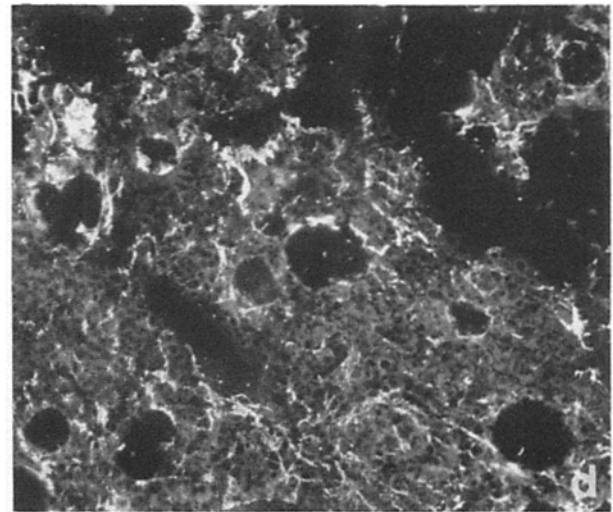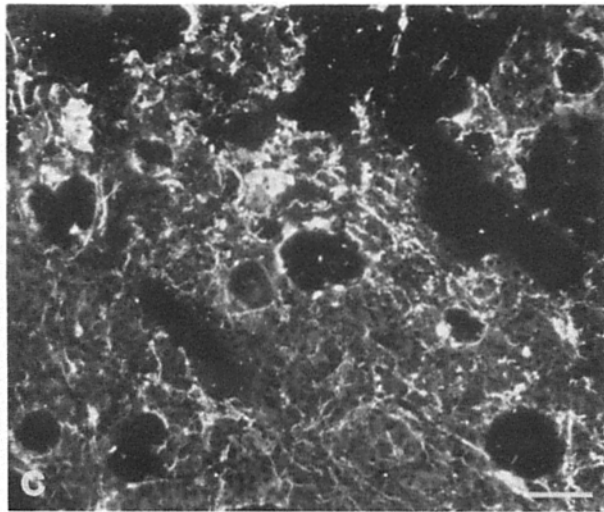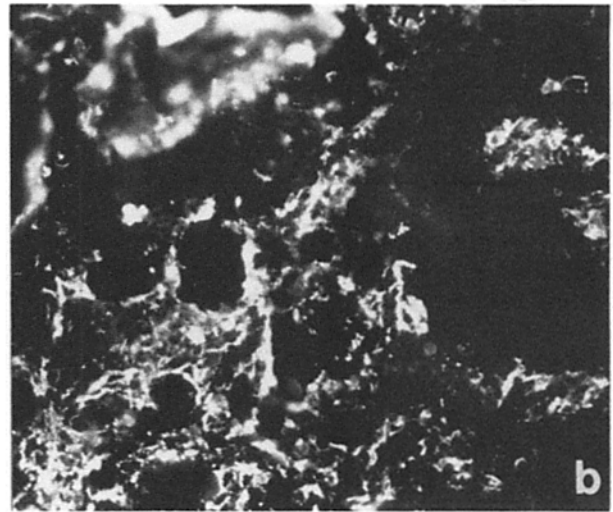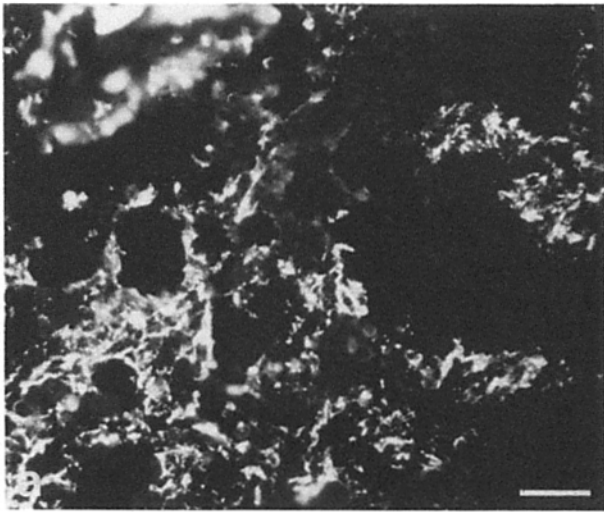
Figure 4. Distribution of tenascin compared with other molecules in the BM. Double immunofluorescence labeling of cryostat sections of human BM are shown. The staining was performed with the antiserum against tenascin (*a, c,* and *e*) and with antibodies against fibronectin (*b*), collagen type III (*d*), and β1-integrin (*e*). The extracellular tenascin which is found between the hematopoietic cells colocalizes both with fibronectin (*a* and *b*) and collagen type III (*c* and *d*). On the other hand the double labeling of tenascin with the monoclonal antibody against the β1-chain of integrins shows no absolute congruence of these two molecules. Tenascin can be seen strongly expressed at sites where the β1-chain is hardly visible. On the other hand, β1-chain expression is also found at sites where tenascin is not deposited (*arrowhead*). Bars, 50 μm.
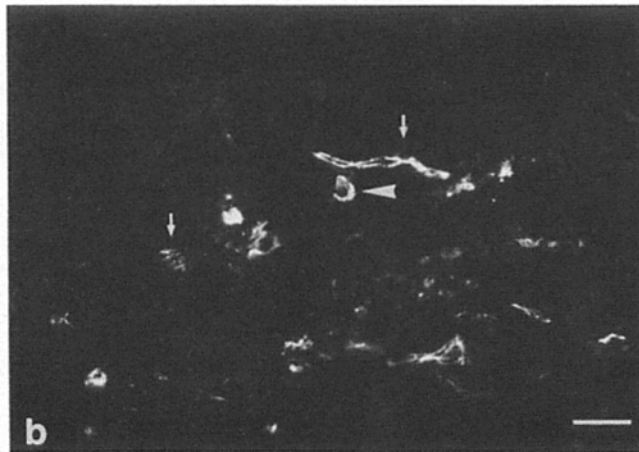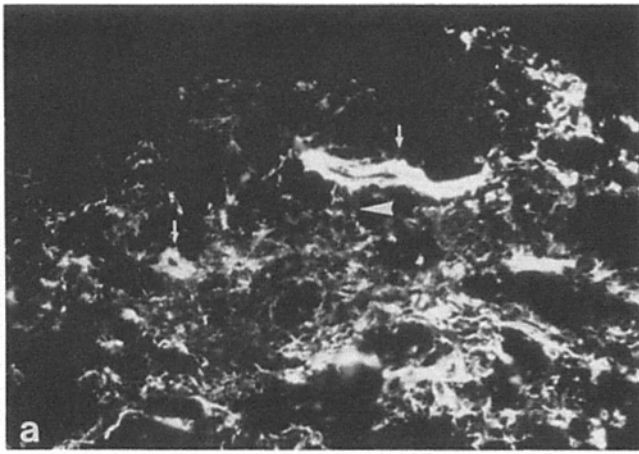
Figure 5. Association of tenascin with blood vessel walls. The double immunofluorescence staining with the endothelial specific monoclonal antibody Rb10 (*a*) and tenascin (*b*) in the cryostat section shows a strong signal in the smooth muscle layer of the arterial vessel walls. This is seen in a longitudinal as well as in a cross section (*arrows*). The sinusoids which are not surrounded by a smooth muscle layer show a much weaker association with tenascin (*arrowhead*). Bar, 50 μm.

antiserum, but with the anti-fibronectin antiserum also some BFU-E could be detected suggesting different modes of action of the two antisera used.

Attachment of Hematopoietic Cells to Immobilized Tenascin

The inhibition experiments using the anti-tenascin antibodies represent an indirect method to determine a cytoadhesive function of tenascin. To show an adhesive property of this molecule in a direct way we purified human tenascin from the conditioned medium of the SK-MEL 28 melanoma cell line according to a procedure recently published by Saginati et al. (37). The higher molecular mass form of tenascin was mainly produced by the melanoma cells but also a weak signal of the lower molecular mass form could be detected by immunoblotting (Fig. 6 *a*). Fibronectin which is also shed in large amounts into the culture medium is efficiently removed by the gelatin-sepharose column. This was evaluated by immunoblotting of 50 μg of our tenascin preparation with an anti-fibronectin antiserum. No fibronectin signal could be found, suggesting that if there is any contamination with fibronectin, it is less than 1:10,000, since we are able to detect five nanograms of purified fibronectin by immunoblotting with the anti-fibronectin antiserum.

2 μg of the tenascin preparation were spotted onto plastic dishes and allowed to air-dry. In Fig. 6, *b* and *c*, the specific binding of the hematopoietic cell lines HL60 and K562 to the immobilized, purified matrix components is shown. Only a weak background binding is seen outside the tenascin-coated areas. Freshly isolated, unfractionated BM cells also attached to tenascin but not as strong as the leukemic cell lines. We compared the binding properties of the heterogeneous BM cells and the homogeneous cell lines to tenascin and to purified fibronectin by counting attached cells to representative mm². Only a quantitative, but not a qualitative, difference of the adhesive capacity could be found (Table II).

Discussion

In the adult organism, expression of tenascin is not ubiqui-

Table I.

Donor number	Number of colonies per 3 × 10⁵ cells‡	Stromal nonadhering progenitors: Number of colonies formed*		% Inhibition§ (difference b–a)
		a) Without antibody treatment	b) With α-tenascin antibody treatment or control antibody treatment	
1	207	79 (38.4%)	110 (53.1%)	14.7%
2	340	7 (2.0%)	47 (13.8%)	11.8%
3	320	10 (3.1%)	44 (13.6%)	10.5%
4	222	38 (16.8%)	68 (30.6%)	13.8%
CONTROLS				
1.1	207	79 (38.4%)	79 (38.4%)	0%
4.1	222	38 (16.8%)	69 (31%)	14.2%

Inhibition of progenitor cell attachment to BM stroma by anti-tenascin antibodies (1–4): evaluation of the nonadherent precursors in mixed committed progenitor colony assays. The results of four individual experiments (1–4) and appropriate control experiments (1.1: anti-laminin; 4.1: anti-fibronectin) are shown.
* Numbers of colonies counted in duplicate assays.
‡ Determination of the total progenitor cell number.
§ Difference of the median percent colony formation in assays with and without antibody treatment calculated as the number of colonies grown from nonadhering cells after incubation on stromal layer divided by the total number of input progenitor cells times 100.
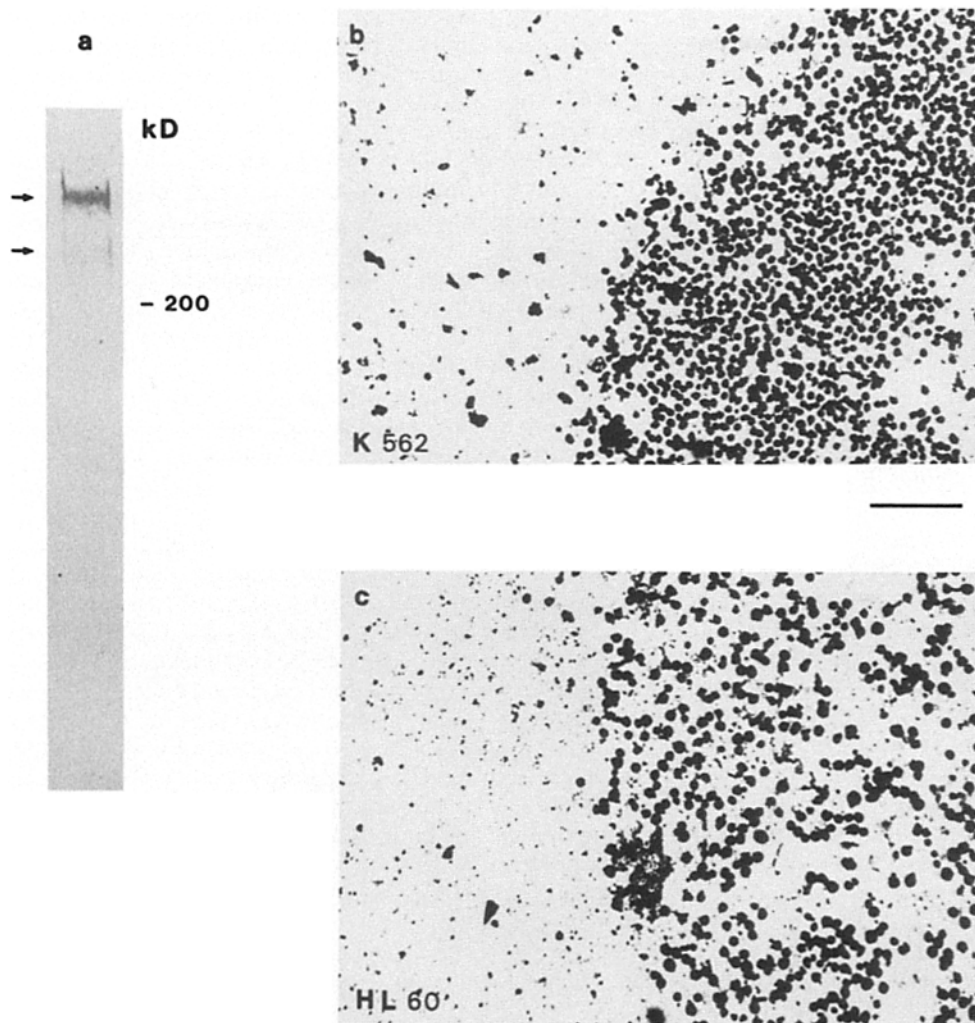
Figure 6. Cell attachment of leukemic lines to purified tenascin. Human tenascin purified from the SK-MEL 28 cell line consists mainly of the higher molecular mass form as shown by immunoblotting (*a*). K562 cells (*b*) and HL60 cells (*c*) were allowed to bind to immobilized human tenascin. After washing, fixation and staining, the border between serum albumin-coated and the tenascin-coated areas is precisely outlined by specifically attached cells. Bar, 50 μm.

tous but confined to certain tissues and organs (9). The present study shows that in the hematopoietic organ tenascin is a constitutive component of its ECM. The BM represents a constantly regenerating organ system containing stem cells. In two other stem cell containing organs, the skin and the intestine, tenascin has also been found to be strongly expressed, suggesting a possible role for tenascin in such proliferative systems. Whereas the function of tenascin in skin and intestine is unknown and only speculative, we now provide evidence for an adhesive function of tenascin for hematopoietic progenitor cells in the human BM.

The large disulfide-linked, six-armed molecular complex of tenascin shows a spider-like appearance in rotary shadowing (18). Each arm of this complex is formed by one subunit

of tenascin which may vary in length due to differentially spliced mRNA variants. The subunits show a modular structure consisting of a constant number of EGF-like repeats, variant fibronectin type III-like repeats and a fibrinogen-like sequence. Two human splice variants corresponding to the six and eight kb messages were reported (23, 31); one subunit with all fifteen fibronectin type III repeats included ("full length") and another form which lacks the type III repeats 6-12. Northern blot analysis for tenascin expression of the stromal cells in long term BM cultures also revealed two mRNA bands of six and eight kb. By sequencing different cDNA clones and using PCR analysis, however, additional isoforms were reported by Siri et al. (41). One splice variant only lacked the fibronectin type III repeat 11,

Table II.

Cell attachment				
	K562	HL60	KG1a	Bone marrow cells
Tenascin	+++	+++	++	++
Fibronectin	+++	+	+	+++

+++, strong binding (>1500 cells/mm²)
++, intermediate binding (500–1500 cells/mm²)
+, moderate binding (100–500 cells/mm²)

whereas in another variant the units 6–9 and 11 were omitted. The corresponding messages of these two additional isoforms, however, could not be differentiated from the six and eight kb mRNA-related variants from the available Northern data. Thus, PCR analysis has to be performed to study the expression of additional mRNA isoforms in BM stromal cells. The protein data obtained from the immunoblot analysis paralleled the mRNA data. Two prominent bands, a stronger band of ~280 kD and a weaker band of 220 kD, could be observed in extracts from BM stromal cells. This result is in accordance with the stronger expression of the 8-kb message. But again it is not possible to decide if the bands detected in the Western blot contain only one or more isoforms of tenascin.

Using fragments of tenascin, it was convincingly demonstrated that the intact tenascin molecule contains two counteracting active sites within one subunit, a cell-binding site and one site responsible for an anti-adhesive signal (42). The functions of the various isoforms of tenascin are not known. However, the cell-binding site and the anti-adhesive site do not seem to be affected by the splicing process because the type III repeats containing the binding sequence and the anti-adhesive sequence are not located within the splice region. Thus, both activities are present within one tenascin molecule and may be used in a cell-type and tissue-specific manner (10). A strong binding of the leukemic cell lines K562 and HL60 and an intermediate binding of the cell line KG1a, which are all thought to represent defined developmental stages, to purified tenascin could be observed. Unfractionated BM cells which, however, represent a mixture of many maturation stages, showed also an intermediate binding. It will be important to determine exactly which progenitor populations can interact with tenascin. Such an approach is feasible by FACS separation of BM cells. In the BM with its high turnover and constant migration of cells, a strong binding of the maturing hematopoietic cells to the microenvironment is not desired. The cells have to attach as well as to be released from the surrounding matrix. The observed correlation between tenascin and fibronectin expression in the extracellular network of native BM sections may indicate that binding strength could be regulated by an interaction of tenascin and fibronectin. Fibroblasts can also attach to tenascin, but tenascin is also able to inhibit cell binding to fibronectin, an effect which can be shown for fibroblasts as well as for monocytes (12, 35). In addition to the attachment assay using purified tenascin, our antibody inhibition assay for cell binding between hematopoietic progenitor cells and the matrix produced by microenvironmental stromal cells also demonstrated that tenascin has binding properties, albeit only moderate for myelomonocytic progenitor cells. But it is clear that tenascin is not the only component responsible for this cell adhesion, and other matrix or cell adhesion molecules are certainly also involved in this process.

In addition to fibronectin, there is also a codistribution of tenascin seen in BM cryostat sections with collagen type III fibers. These interstitial fibers are thought to play a role in the mechanical stability of the tissue, whereas the noncollagenous components fibronectin and tenascin might coordinate cell adhesion and migration. Tenascin can also be found in the walls of the blood vessels consistent with previous studies which have documented an association of tenascin expression with smooth muscle cells (1, 29).

Only sparse information is available concerning possible cellular receptors for tenascin. In a previous report, a member of the β1-integrin family has been suggested to act as a cell-membrane bound tenascin ligand (4). In BM cryostat sections, an exact congruence of the β1-integrin chain and tenascin expression was not detected, but this observation does not rule out that a member of the β1-integrins can act as a ligand on hematopoietic cells for tenascin in this tissue. There may be different receptors present for tenascin in the hematopoietic system. Another component which has been reported to bind to tenascin is syndecan, a transmembrane proteoglycan. Different forms of syndecan exist, but only the mesenchymal syndecan with heparan sulfate glycosaminoglycan side chains interacts strongly with tenascin (38). Unfortunately there are no data available concerning syndecan expression on stromal cells. Analysis of this receptor type will reveal if a proteoglycan-tenascin interaction plays a role in the hematopoietic process.

To date there is not much information about the specific attachment of the different committed progenitor cells and stem cells homing to the BM microenvironment. Only hemonectin is expressed in the BM in a tissue-restricted fashion and shows a lineage specificity for maturing granulocytes (7). Fibronectin and thrombospondin are also known to function as adhesive components in the BM, but the ubiquitous distribution of these components raises doubts about a specific involvement in progenitor cell binding. Tenascin has a more restricted expression pattern than fibronectin but is also not specific for the BM. It may well be that combinations or interactions of different ECM components will lead to an appropriate specific microenvironment within the hematopoietic system. Attachment studies using specifically enriched progenitor subpopulations with purified tenascin and combinations with other extracellular components will allow us to define more precisely the role of tenascin in the binding process of maturing hematopoietic cells.

References

1. Aufderheide, E., and P. Ekblom. 1988. Tenascin during gut development: appearance in the mesenchyme, shift in molecular forms and dependence on epithelial-mesenchymal interactions. *J. Cell Biol.* 107:2341–2349.
2. Aufderheide, E., R. Chiquet-Ehrismann, and P. Ekblom. 1987. Epithelial-mesenchymal interactions in the developing kidney lead to expression of tenascin in the mesenchyme. *J. Cell Biol.* 105:599–608.
3. Bentley, S. A. 1981. Close range cell-cell interaction required for stem cell maintenance in long term bone marrow culture. *Exp. Hematol.* 9:308–312.
4. Bourdon, M. A., and E. Ruoslahti. 1989. Tenascin mediates cell attachment through an RGD-dependent receptor. *J. Cell Biol.* 108:1149–1155.
5. Campbell, A. D., and M. S. Wicha. 1988. Extracellular matrix and the hematopoietic microenvironment. *J. Lab. Clin. Med.* 112:140–146.
6. Campbell, A. D., M. W. Long, and M. S. Wicha. 1987. Haemonectin, a bone marrow adhesion protein specific for cells of the granulocytic lineage. *Nature (Lond.).* 329:744–746.
7. Campbell, A. D., M. W. Long, and M. S. Wicha. 1990. Developmental regulation of granulocytic cell binding to hemonectin. *Blood.* 76:1758–1764.
8. Carnemolla, B., L. Borsi, G. Bannikov, S. Troyanovsky, and L. Zardi.

1992. Comparison of human tenascin expression in normal, Simian-virus-40-transformed and tumor-derived cell lines. *Eur. J. Biochem.* 205:561–567.

9. Chiquet-Ehrismann, R. 1990. What distinguishes tenascin from fibronectin? *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2598–2604.

10. Chiquet-Ehrismann, R. 1991. Anti-adhesive molecules of the extracellular matrix. *Curr. Opin. Cell Biol.* 3:800–804.

11. Chiquet-Ehrismann, R., E. J. Mackie, C. A. Pearson, and T. Sakakura. 1986. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell.* 47:131–139.

12. Chiquet-Ehrismann, R., P. Kalla, C. A. Pearson, K. Beck, and M. Chiquet. 1988. Tenascin interferes with fibronectin action. *Cell.* 53:383–390.

13. Dexter, T. M., E. Spooncer, R. Schofield, B. I. Lord, and P. Simmons. 1984. Haemopoietic stem cells and the problem of self-renewal. *Blood Cells (Berl.).* 10:315–339.

14. Dexter, T. M., E. Spooncer, P. Simmons, and T. D. Allen. 1984. Long-term marrow culture: an overview of techniques and experience. *In* Long-term Bone Marrow Culture. D. G. Wright and J. S. Greenberger, editors. Alan R. Liss., Inc. 57–96.

15. Dexter, T. M., L. H. Coutinho, E. Spooncer, C. M. Heyworth, C. P. Daniel, R. Schiro, J. Chang, and T. D. Allen. 1990. Stromal cells in haemopoiesis. Molecular control of haemopoiesis. *Ciba Found. Symp.* 148:76–95.

16. Engler-Blum, G., M. Meier, J. Frank, and G. A. Müller. 1993. Reduction of background problems in nonradioactive Northern and Southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations. *Anal. Biochem.* 210:235–244.

17. Erickson, H. P., and M. A. Bourdon. 1989. Tenascin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annu. Rev. Cell Biol.* 5:71–92.

18. Erickson, H. P., and V. A. Lightner. 1988. Hexabrachion protein (tenascin, cytotactin, brachionectin) in connective tissues, embryonic brain, and tumors. *Adv. Cell Biol.* 2:55–90.

19. Gartner, S., and H. S. Kaplan. 1980. Long term culture of human bone marrow cells. *Proc. Natl. Acad. Sci. USA.* 77:4756–4759.

20. Giancotti, F. G., P. M. Comoglio, and G. Tarone. 1986. Fibronectin-plasma membrane interaction in the adhesion of hematopoietic cells. *J. Cell Biol.* 103:429–437.

21. Gordon, M. Y. 1988. Adhesive properties of haematopoietic stem cells. *Br. J. Haematol.* 68:149–151.

22. Gordon, M. Y., G. P. Riley, S. M. Watt, and M. F. Greaves. 1987. Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature (Lond.).* 326:403–405.

23. Gulcher, J. R., D. E. Nies, L. S. Marton, and K. Stefansson. 1989. An alternatively spliced region of the human hexabrachion contains a repeat of potential N-glycosylation sites. *Proc. Natl. Acad. Sci. USA.* 86:1588–1592.

24. Hoffman, S., K. L. Crossin, F. S. Jones, D. R. Friedlander, and G. M. Edelman. 1990. Cytotactin and cytotactin-binding proteoglycan. An interactive pair of extracellular matrix proteins. *Ann. NY. Acad. Sci.* 580:288–301.

25. Klein, G., M. Langegger, R. Timpl, and P. Ekblom. 1988. Role of laminin A chain in the development of epithelial cell polarity. *Cell.* 55:331–341.

26. Lightner, V. A., F. Gumkowski, D. D. Bigner, and H. P. Erickson. 1989.

Tenascin/hexabrachion in human skin: biochemical identification and localization by light and electron microscopy. *J. Cell Biol.* 108:2483–2493.

27. Long, M. W., and V. M. Dixit. 1990. Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. *Blood.* 75:2311–2318.

28. Mackie, E. J., W. Halfter, and D. Liverani. 1988. Induction of tenascin in healing wounds. *J. Cell Biol.* 107:2757–2767.

29. Matsuoka, Y., J. Spring, K. Ballmer-Hofer, U. Hofer, and R. Chiquet-Ehrismann. 1990. Differential expression of tenascin splicing variants in the chick gizzard and in cell cultures. *Cell Differ. Develop.* 32:417–424.

30. Natali, P. G., M. R. Nicotra, A. Bigotti, P. Castellani, A. M. Risso, and L. Zardi. 1991. Comparative analysis of the expression of the extracellular matrix protein tenascin in normal human fetal, adult and tumor tissues. *Int. J. Cancer.* 47:811–816.

31. Nies, D. E., T. J. Hemesath, J. H. Kim, J. R. Gulcher, and K. Stefansson. 1991. The complete cDNA sequence of human hexabrachion (tenascin). A multidomain protein containing unique epidermal growth factor repeats. *J. Biol. Chem.* 266:2818–2823.

32. Patel, V. P., and M. F. Lodish. 1984. Loss of adhesion of murine erythroleukemia cells to fibronectin during erythroid differentiation. *Science (Wash. DC).* 224:996–998.

33. Patel, V. P., and M. F. Lodish. 1986. The fibronectin receptor on mammalian erythroid cells: characterization and developmental regulation. *J. Cell Biol.* 102:449–456.

34. Roberts, R., J. Gallagher, E. Spooncer, T. D. Allen, F. Bloomfield, and T. M. Dexter. 1988. Heparan sulfate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature (Lond.).* 332:376–378.

35. Rüegg, C. R., R. Chiquet-Ehrismann, and Ş. Ş. Alkan. 1989. Tenascin, an extracellular matrix protein, exerts immunomodulatory activities. *Proc. Natl. Acad. Sci. USA.* 86:7437–7441.

36. Saga, Y., T. Yagi, Y. Ikawa, T. Sakakura, and S. Aizawa. 1992. Mice develop normally without tenascin. *Genes & Dev.* 6:1821–1831.

37. Saginati, M., A. Siri, E. Balza, M. Ponassi, and L. Zardi. 1992. A simple procedure for tenascin purification. *Eur. J. Biochem.* 205:545–549.

38. Salmivirta, M., K. Elenius, S. Vainio, U. Hofer, R. Chiquet-Ehrismann, I. Thesleff, and M. Jalkanen. 1991. Syndecan from embryonic tooth mesenchyme binds tenascin. *J. Biol. Chem.* 266:7733–7739.

39. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 7.19–7.43.

40. Singer, J. W., A. Keating, and T. N. Wight. 1985. The human hematopoietic microenvironment. *In* Recent Advances in Haematology. A. V. Hofmann, editor. Churchill-Livingstone, Inc., New York. 1–24.

41. Siri, A., B. Carnemolla, M. Saginati, A. Leprini, G. Casari, F. Baralle, and L. Zardi. 1991. Human tenascin: primary structure, pre-mRNA splicing patterns and localization of the epitopes recognized by two monoclonal antibodies. *Nucleic Acid Res.* 19:525–531.

42. Spring, J., K. Beck, and R. Chiquet-Ehrismann. 1989. Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell.* 59:325–334.

43. Thesleff, I., E. Mackie, S. Vainio, and R. Chiquet-Ehrismann. 1987. Changes in the distribution of tenascin during tooth development. *Development.* 101:289–296.

44. Weller, A., S. Beck, and P. Ekblom. 1991. Amino acid sequence of mouse tenascin and differential expression of two tenascin isoforms during embryogenesis. *J. Cell Biol.* 112:355–362.