Hydrocortisone regulates types I and III collagen gene expression and collagen synthesis in human marrow stromal cells

MIREYA FERNÁNDEZ* and JOSÉ J MINGUELL

Unidad de Biología Celular, INTA, Universidad de Chile, Santiago, Chile

Hematopoiesis is the resultant of the orderly molecular and cellular interactions between progenitor cells and stroma. ***In vitro*** *studies (Dexter-type cultures) have shown that initiation of hematopoiesis only occurs after establishment of a hydrocortisone-dependent layer of stromal cells. Although the molecular basis for the requirement of hydrocortisone are not well understood, data have shown that synthesis/assembly of extracellular matrix molecules (proteoglycans and fibronectin) is regulated by hydrocortisone. Since interstitial collagens are abundantly expressed in the marrow stroma, we investigated whether hydrocortisone may also modulate the expression of collagen types I and III. For these studies, human bone marrow fibroblast cultures were grown in standard culture medium either in the absence or presence of 10^{-7} M hydrocortisone. Under both conditions, bone marrow fibroblasts synthesized collagen types I and III, and expressed the respective genes. However, hydrocortisone produced a decrease in the synthesis of interstitial collagens and also in the relative abundance of pro-α_1(I) and pro-α_1(III) mRNAs. The results of this study are consistent with the assumption that glucocorticoids regulate the expression of several extracellular matrix molecules in the marrow stroma and thus permit* ***in vitro*** *hematopoiesis to occur.*

Key terms*: collagen, fibroblasts, gene expression, hydrocortisone, marrow stroma.*

INTRODUCTION

Several studies both *in vivo* and *in vitro* have been undertaken to define the biological properties of stromal cells of the hemopoietic microenvironment. *In vitro*, the technique of long-term marrow culture and the subsequent utilization of the adherent stromal layer produced, as well as the derivation of clonal stromal cell lines, have greatly aided research on the nature and properties of the marrow stroma (Dexter, 1982, Greenberger, 1991). Together, these studies have indicated that several stromal extracellular matrix (ECM) molecules are involved in the homing of the hemopoietic progenitor cell, as well as in the capture of growth factors (Tavassoli & Minguell, 1991). In the bone marrow, stromal cells produce several types of ECM molecules, among them various types of collagen (Bentley, 1982; Waterhouse *et al*, 1986). Although the role of collagen in hemopoiesis has not been elucidated, the observation that the *in vitro* proliferation and maturation of hemopoietic precursors is decreased

* **Correspondence to:** Dr Mireya Fernández, Unidad de Biología Celular, INTA, Universidad de Chile, Casilla 138, Santiago 11, Chile. Phone: (56-2) 678-1428. Fax: (56-2) 221-4030. E-mail: jminguel@uec.inta.uchile.cl

by proline analogs (Zuckerman *et al*, 1985), emphasized a role for collagen in the maintenance of hemopoiesis.

It has been documented that *in vitro* hematopoiesis (Dexter-type long-term cultures) only occurs after supplementing the culture medium with glucocorticoids. Under these conditions, stromal cells proliferate and maintain the self-replication of myeloid progenitor cells (Sakakeeny & Greenberger, 1982), while in the absence of hydrocortisone (HC), bone marrow stromal cells proliferate but do not maintain granulopoiesis (Johnson & Dorshkind, 1986).

Several studies have shown that glucocorticoids induce changes in the structure of stromal ECM by affecting the production or assembly of proteoglycans (Bentley *et al*, 1988; Morris *et al*, 1991) and/or fibronectin (McKeown-Longo & Etzler, 1987). Whether the production of collagen by marrow stromal cells is also modulated by glucocorticoids, as it occurs in other tissues (Hamalainen *et al*, 1985; Slack *et al*, 1993) has not been well established. Since interstitial collagens play a central role in hematopoiesis (Klein, 1995; Fernández & Minguell, 1996), we studied the effect of hydrocortisone on the capacity of human bone marrow stromal cells to express genes for collagen types I and III, as well as in the production of interstitial collagens. The results obtained indicate that the extent of type I and III collagen synthesis as well as the level of expression of their respective mRNA's were lower for cells maintained in the presence than in the absence of hydrocortisone.

METHODS

Bone marrow fibroblasts cultures

Bone marrow from normal donors (4 to 12 years of age) was collected under protocols approved by the institutional board of the Children's Hospital, Santiago, Chile. Bone marrow fibroblasts (BMF) cultures were prepared as previously described (Chichester *et al*, 1993) and cultured in the absence or presence of 10^{-7} M hydrocortisone. After one week in culture, adherent fibroblast-like cells were released from the culture dishes by trypsinization and replated three times in the respective culture medium. Cells thus obtained were used to study collagen gene expression and collagen synthesis.

All tissue culture reagents used were from Gibco Life Technologies, Baltimore, MD.

Collagen synthesis

To study collagen synthesis, BMF cultures which have reached confluence ($2.5x10^5$ cells/cm^2) were labeled for 24 h at 37°C with 10 mCi/ml of ^{3}H-proline (20 Ci/mmol; Amersham Corp, Arlington Heights, IL) in a-MEM medium containing 1% FCS, 50 mg/ml each of ascorbic acid and β-aminopropionitrile. After the labeling period, the culture medium was harvested and aliquots were taken for analysis of collagen and non-collagen proteins using a collagenase-sensitive material assay (Peterkofsky & Diegelmann, 1971). To characterize the procollagen chains, aliquots of labeled culture medium were precipitated with ammonium sulfate (300 mg/ml), dissolved and dialyzed in a 25 mM Tris-HCl buffer containing 2 M urea and 1 mM EDTA, pH 7.5 (Booth *et al*, 1980). The dialyzed materials were applied to DEAE-cellulose columns (2.2 x 16 cm), which were equilibrated with the dialysis buffer. The columns were eluted by a linear gradient (up to 0.2 M) of NaCl prepared in the same buffer. Fractions were collected, radioactivity measured and collagen content estimated by the collagenase-sensitive material assay. The type of procollagen chains was determined by SDS-PAGE (Laemmli, 1970), using collagen type I and III as standards and Coomassie blue for staining. All chemicals used were from Sigma Chemical Co, St Louis, MO.

Northern blot analysis

For Northern hybridization studies, RNA was isolated as described (Chomczynski & Sacchi, 1987) and RNA integrity was assessed by UV shadowing (Nohtomi *et al*, 1994). Poly $(A)^+$ RNA was purified using oligo dT chromatography. After measuring RNA content (OD_{260} absorbance), samples were separated on 0.9% agarose gels under

denaturing conditions and transferred onto nylon membranes (Sigma Chemical Co, St Louis, MO). After immobilization and prehybridization, the membranes were hybridized with one of the sequence specific cDNA probes (see below) labeled with (^{32}P) (800 Ci/mmol; Amersham Corp, Arlington Heights, IL) by random prime labeling (Feingberg & Volgelstein, 1983). After hybridization, the membranes were washed, air-dried and exposed to x-ray films (Kodak XAR-2, Eastman Kodak Co, Rochester, NY). The Northern blot autoradiograms were quantitated by using a scanning laser densitometer interfaced with an integrator. Densitometric units for each probe were corrected for β-actin expression, as previously indicated (Chichester *et al*, 1993).

The following cDNA probes were used: a 1.5-kb pro α_1 (I) (Hf677) cDNA for type I collagen (Chu *et al*, 1982) and a 2.4-kb pro α_1 (III) (E6) cDNA for type III collagen (Loidl *et al*, 1984). Two antisense oligomeric probes (ACAGCTTCTCCTTAA TGTCACGCACGATTT and CACTTCAT GATGGAGTTGAAGGTAGTTTCG) were used for β-actin. Plasmid Hf-677 was obtained from the American Type Culture Collection, Rockville, MD. Plasmid E6 was kindly provided by Dr Jeanne C Myers, University of Pennsylvania, Philadelphia, PA.

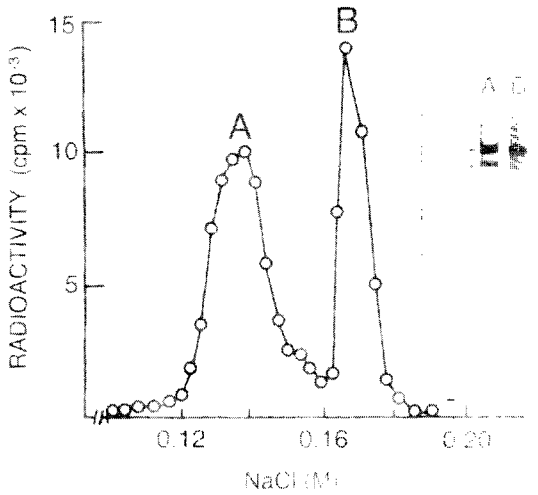

Fig 1. Separation by DEAE-cellulose chromatography of ^{3}H-labeled procollagens produced by bone marrow fibroblasts. After labeling BMF cultures with ^{3}H-proline, the medium was saved, precipitated by ammonium sulfate (300 mg/ml) and applied to a DEAE-cellulose column. Labeled peaks A and B were obtained after eluting the column with a linear gradient of NaCl (up to 0.2 M). The collagenase-sensitive material in each peak was further analyzed by SDS-PAGE to reveal the nature of the procollagen chains. Inset, SDS-PAGE pattern (7.5%, reduced) of procollagen material in peaks A and B. The migration position for each procollagen chain is indicated: in A, α_1(I) and α_2 (I); in B, α_1 (III).

RESULTS

To analyze the nature of the collagen produced by BMF, cells were radiolabeled with ^{3}H-proline and the collagenous material released to the medium was analyzed by DEAE-cellulose chromatography and SDS-PAGE. For BMF cultivated in the absence of hydrocortisone (control), DEAE-cellulose chromatography (Fig 1) revealed the presence of two radioactive peaks (A and B) eluting at 0.14 and 0.17 M NaCl, respectively. As assessed by a collagenase-sensitive material assay, more than 90% of the radioactive material on each peak proved to be collagen. SDS-PAGE showed the presence of α_1 (I) and α_2 (I) procollagen chains which associated to peak A, whereas α_1 (III) procollagen chains associated to peak B (inset, Fig 1). The patterns in DEAE-cellulose and in SDS-PAGE were similar for both BMF cultured in the presence of HC and control cells (data not shown).

The effect of hydrocortisone on interstitial collagen synthesis by BMF was measured and results are shown in Table I. The synthesis of collagen by HC-treated cells was 3-fold lower than that exhibited by HC-untreated (control) cells. Similarly, the relative rate of collagen production was lower (2.3-fold) in HC-treated cells. The production of non-collagen proteins, which comprise a broad group of collagenase-resistant labeled proteins, was also lower (1.3-fold) in HC-treated than in HC-untreated cells.

BMF cultured under both conditions were used to study their ability to express genes encoding for types I and III collagen. RNA was fractionated by electrophoresis and analyzed by Northern hybridization with cDNAs probes for interstitial collagen genes. As seen in Figure 2, BMF cultured either in the absence or presence of HC exhibited hybridization signals for pro-α_1 (I) mRNA (as represented by two characteris-

Table I

Synthesis of collagen by bone marrow fibroblasts.

Condition	Collagen (cpm)*	Non collagen (cpm)*	Relative rate**
-HC	5,750 ± 50	54,350 ± 775	1.93 ± 0.05
+HC	1,915 ± 450	42,000 ± 1,082	0.83 ± 0.20

To measure collagen and non-collagen protein synthesis, BMF ($5x10^5$ cells/well) cultures grown in the absence (-HC) and in the presence (+HC) of hydrocortisone were labeled for 24 h with ^{3}H-proline.

* Culture media were saved and used to measure the ^{3}H-radioactivity incorporated into collagenase-sensitive (collagen) and collagenase-resistant (noncollagen) proteins.

** The relative rate of collagen production was calculated from the formula:
^{3}H-collagen cpm / (^{3}H-non collagen proteins cpm x 5.4 + ^{3}H-collagen cpm) x 100.
Values, means ± SEM's of at least 3 triplicate experiments.

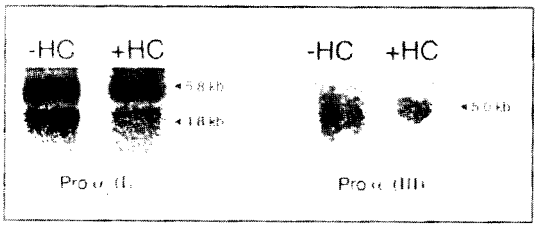

Fig 2. Northern blot analysis of bone marrow fibroblast collagen types I and III mRNA. Northern blots of 0.5 µg poliA$^+$ RNA each, obtained from BMF cultures grown in the absence (-HC) and in the presence (+HC) of hydrocortisone. Arrows indicate apparent molecular size (kb) of each transcript.

tic transcripts of 5.8 and 4.8 kb) and for pro-α_1 (III) mRNA (represented as a broad band at approximately 5.0 kb). The intensity of the bands showed differences between HC-untreated and HC-treated cells.

The relative abundance of each mRNA probe was assessed after digitalization, integration and correction for β-actin expression, The results are shown in Table II. The relative abundance of collagen pro-α_1 (I) mRNA decreased by 50% when cells were treated with hydrocortisone. On the other side, the relative abundance of collagen pro-α_1 (III) mRNA, decreased by 92% after treatment of cells with HC.

DISCUSSION

In vitro studies of hemopoiesis have shown that the proliferation of progenitor cells depends on the development of an adherent layer of marrow stromal cells, which produces several cytokines and ECM molecules (Verfaillie *et al*, 1994). The establishment of the adherent layer of stromal cells, as well as its ability to sustain hemopoiesis, is strongly dependent on the culture conditions used to grow the cells. As mentioned, when stromal cells are grown in the presence of hydrocortisone, they become hematocompetent and sustain the proliferation and differentiation of progenitor cells (Sakakeeny & Greenberger, 1982). Whether HC affects the proliferative/differentiative capacity of progenitor cells, or their ability to establish adhesive interactions with stroma, has not been well established (Thalmeier *et al*, 1996). However, there is growing evidence that the nature of ECM produced by marrow stroma is modulated by glucocorticoids. First, the treatment of stromal cells with hydrocortisone results in a shift of the production and

Table II

Collagen gene expression by bone marrow fibroblasts

	Relative abundance of mRNA	
	-HC	+HC
Collagen pro α_1 (I)	0.10	0.050
Collagen pro α_1 (III)	0.32	0.027

Northern blots of total RNA (10 µg each), prepared from hydrocortisone untreated (-HC) and treated (+HC) BMF cultures, were processed for digitalization and integration. Data are expressed as densitometric absorbance units after β-actin correction. Similar results were obtained in two separate experiments.

molecular nature of stromal proteoglycans (Bentley *et al*, 1988), that participate in hemopoietic stem cell homing (Tavassoli & Minguell, 1991). Second, it has been shown that methylprednisolone changes the molecular structure of heparan sulfate on the surface of stromal cells (Siczkoswski *et al*, 1993), with a concomitant effect on the ability of the marrow stroma to bind and stimulate the differentiation of progenitor cells (Gordon *et al*, 1988; Minguell, 1993). Third, glucocorticoids are involved in the induction of fibronectin-matrix assembly (McKeown-Longo & Etzler, 1987).

In addition, our data show that both in the absence or in the presence of HC, bone marrow stromal cells produce mRNA for collagen chains pro-α_1 (I) and pro-α_1 (III). However, the relative abundance of pro-α_1 (I) and pro-α_1 (III) mRNAs decreased in HC-treated cells, being the effect of the glucocorticoid more pronounced for pro-α_1 (III) than for pro-α_1 (I) gene expression. This effect of glucocorticoids seems not to be specific for marrow stromal cells, since the expression of interstitial collagen genes is also down-regulated by HC in stromal cells from other tissues (Slack *et al*, 1993; Delany *et al*, 1995a).

Similarly, the incorporation of ^{3}H-proline into collagenase-sensitive proteins (Table 1) was also lower in cells grown in the presence of hydrocortisone. It was not further investigated if changes in ^{3}H-proline incorporation were the consequence of alterations in the rate of synthesis or degradation (Brinckerhoff & Auble, 1990; Delany *et al*, 1995b) of collagen. The observed reduction in collagen synthesis by glucocorticoids is not without precedent, since HC produced similar effects in other tissues (Delany *et al*, 1995b; Guller *et al*, 1995).

Thus, present results are further evidence to show that the pattern of expression of various ECM molecules is modulated by glucocorticoids. Therefore, the expression of a permissive microenvironment for hemopoiesis seems to be the resultant of a balance in the production and assembly of ECM by stromal cells, which seems to be finely tuned by a variety of stimuli (Raghow, 1994). The observed effect of hydrocortisone on collagen synthesis, as well as in other ECM molecules, may then explain why HC is required for long-term hemopoiesis (Dexter, 1982; Sakakeeny & Greenberger, 1982) or to "prime" marrow stroma to improve hemopoiesis after recharging with isolated progenitor cells (Liesveld *et al*, 1993).

ACKNOWLEDGEMENTS

This work was supported by grants from FONDECYT-Chile (195-0238) and Fundaçao VITAE-Brazil (B 11487/4B001).

REFERENCES

BENTLEY SA (1982) Collagen synthesis by bone marrow stromal cells: a quantitative study. Br J Haematol 50: 491-497

BENTLEY A, KIRBY L, ANKLESARIA P, GREENBERGER JS (1988) Bone marrow stromal proteoglycan heterogeneity: phenotypic variability between cell lines and the effect of glucocorticoid. J Cell Physiol 136: 182-187

BOOTH BA, POLAK KL, UITTO J (1980) Collagen biosynthesis by human skin fibroblasts. I. Optimization of the culture conditions for synthesis of type I and type III procollagens. Biochim Biophys Acta 607: 145-160

BRINCKERHOFF CE, AUBLE DT (1990) Regulation of collagenase gene expression in synovial fibroblasts. Ann NY Acad Sci 580: 355-374

CHICHESTER CO, FERNÁNDEZ M, MINGUELL JJ (1993) Extracellular matrix gene expression by human bone marrow stroma and by marrow fibroblasts. Cell Adh Commun 1: 93-99

CHOMCZYNSKI P, SACCHI N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159

CHU ML, MYERS JC, BERNARD MP, DING DF, RAMIREZ F (1982) Cloning and characterization of five overlapping cDNAs specific for the human pro alpha 1 (I) collagen. Nucleic Acids Res 10: 5925-5934

DELANY AM, GABBITAS BY, CANALIS E (1995a) Cortisol downregulates osteoblast alpha 1 (I) procollagen mRNA by transcriptional and posttranscriptional mechanisms. J Cell Biochem 57: 488-494

DELANY AM, JEFFREY JJ, RYDZIEL S, CANALIS E (1995b) Cortisol increases interstitial collagenase expression in osteoblasts by post-transcriptional mechanisms. J Biol Chem 270: 26607-26612

DEXTER TM (1982) Stromal cell associated hemopoiesis. J Cell Physiol 1: 87-99

FEINGBERG AP, VOLGELSTEIN B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6-13

FERNANDEZ M, MINGUELL JJ (1996) The role of collagen in hematopoiesis. Braz J Med Biol Res 29: 1201-1207

GORDON MY, RILEY GP, CLARKE D (1988) Heparan sulfate is necessary for adhesive interactions between human early hemopoietic progenitor cells and the ex-

tracellular matrix of the marrow microenvironment. Leukemia 2:804-809

GREENBERGER JS (1991) The hematopoietic microenvironment. Crit Rev Oncol Hematol 11: 65-84

GULLER S, KONG L, WOZNIAK R, LOCKWOOD CJ (1995) Reduction of extracellular matrix protein expression in human amnion epithelial cells by glucocorticoids: a potential role in preterm rupture of the fetal membranes. J Clin Endocrinol Metab 80: 2244-2250

HAMALAINEN L, OIKARINEN J, KIVIRIKKO KI (1985) Synthesis and degradation of type I procollagen mRNAs in cultured human skin fibroblasts and the effect of cortisol. J Biol Chem 260: 720-725

JOHNSON A, DORSHKIND K (1986) Stromal cells in myeloid and lymphoid long-term bone marrow cultures can support multiple hemopoietic lineages and modulate their production of hemopoietic growth factors. Blood 68: 1348-1354

KLEIN G (1995) The extracellular matrix of the hematopoietic microenvironment. Experientia 51: 914-926

LAEMMLI UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-681

LIESVELD JL, WINSLOW JM, FREDIANI KE, RYAN DH, ABBOUD CN (1993) Expression of integrins and examination of their adhesive function in normal and leukemic hematopoietic cells. Blood 81: 112-121

LOIDL HR, BRINKER JM, MAY M, PIHLAJANIEMI T, MORROW S, ROSENBAUM J, MYERS JC (1984) Molecular cloning and carboxyl-propeptide analysis of human type III procollagen. Nucleic Acids Res 12: 9383-9394

MCKEOWN-LONGO PJ, ETZLER CA (1987) Induction of fibronectin matrix assembly in human fibrosarcoma cells by dexamethasone. J Cell Biol 104: 601-610

MINGUELL JJ (1993) Is hyaluronic acid the "organizer" of the extracellular matrix in marrow stroma? Exp Hematol 21: 7-8

MORRIS AJ, TURNBULL JE, RILEY GP, GORDON MY, GALLAGHER JT (1991) Production of heparan sulphate proteoglycans by human bone marrow stromal cells. J Cell Sci 99: 149-156

NOHTOMI K, SATO K, SHIZUME K, YAMAZAKI K, DEMURA H, HOSODA K, MURATA Y, SEO H (1994) Stimulation of interleukin-4 of cell proliferation and mRNA expression of alkaline phosphatase and collagen type I in human osteoblast-like cells of trabecular bone. Bone & Mineral 27: 69-79.

PETERKOFSKY B, DIEGELMANN R (1971) Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10: 988-994

RAGHOW R (1994) The role of extracellular matrix in postinflammatory wound healing and fibrosis. FASEB J 8: 823-831

SAKAKEENY MA, GREENBERGER JS (1982) Granulopoiesis longevity in continuous bone marrow cultures and factor dependent cell line generation: significant variation among 28 inbred mouse strains and outbred stocks. J Natl Cancer Inst 68: 305-317

SICZKOSWSKI M, ANDREW T, AMOS S, GORDON MY (1993) Hyaluronic acid regulates the function and distribution of sulfated glycosaminoglycans in bone marrow stromal cells. Exp Hematol 21: 126-130

SLACK JL, LISKA DJ, BORNSTEIN P (1993) Regulation of expression of the type I collagen genes. Am J Med Genet 45: 140-151

TAVASSOLI M, MINGUELL JJ (1991) Homing of hemopoietic progenitor cells to the marrow. Proc Soc Exp Biol Med 196:367-373

THALMEIER K, MEISNER P, REISBACH G, HÜLTNER L, MORTENSEN BT, BRECHTEL A, OOSTENDORP RAJ, DÓRMER P (1996) Constitutive and modulated cytokine expression in two permanent human bone marrow stromal cells lines. Exp Hematol 24:1-10

VERFAILLIE C, HURLEY R, BHATIA R, McCARTHY JB (1994) Role of marrow matrix in normal and abnormal hematopoiesis. Crit Rev Oncol Hematol 16: 201-224

WATERHOUSE E, QUESENBERRY PJ, BALIAN G (1986) Collagen synthesis by murine bone marrow cell culture. J Cell Physiol 127: 397-402

ZUCKERMAN KS, RHODES RK, GOODRUM DD, PATEL VR, SPARKS B, NELL J, WICHA MS, MAYO LA (1985) Inhibition of collagen deposition in the extracellular matrix prevents the establishment of a stroma supportive of hematopoiesis in long-term murine bone marrow cultures. J Clin Invest 75: 970-975