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Original Article

Interleukin-1 and interleukin-6 differentially regulate the accumulation of newly synthesized extracellular matrix components and the cytokine release by developing chick embryo skin fibroblasts

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ABSTRACT In the present study, we demonstrate that both interleukin-1 (IL-1) and interleukin-6 (IL-6) induced a significant decrease in glycosaminoglycan (GAG) synthesis and, more strikingly, secretion by 7 and 13 day-old chick embryo skin fibroblasts. We demonstrated that interleukin treatment also inhibited the synthesis of collagenase-digestible proteins (type I collagen). In addition, tissue culture supernatants (conditioned media, CM) were tested for reactivity for IL specific ELISAs and for their ability to stimulate proliferative responses in mouse thymocytes and hybridoma cells. Our findings demonstrate that chick embryo skin fibroblasts spontaneously produce IL-1 and, in even greater amounts, IL-6. Highest levels of interleukin secretion were found in the CM of 13 day-old fibroblasts and the IL-1ß isoform was predominant over IL-1α. Pretreatment of the fibroblasts with either IL-1 or IL-6 increased the secretion of both cytokines. Increased IL-1 levels were correlated with enhanced IL-1 bioactivity in the CM of IL-6 treated fibroblasts. By contrast, the raised concentrations of IL-1 in the CM of IL-1 treated cells and IL-6 in the CM of IL-1 or IL-6 treated fibroblasts failed to translate into augmented bioactivity. These observations, taken together, indicated that IL-1 and IL-6 are able to regulate the synthesis and secretion of ECM macromolecules of developing connective tissues and the cytokine release by chick embryo skin fibroblasts. We hypothesize that the treatment of fibroblasts with exogenous cytokines may induce, in addition to the cytokines themselves, the secretion of autocrine peptide regulators of growth which act either synergistically or antagonistically with the interleukins.

KEY WORDS: *interleukin-1, interleukin-6, glycosaminoglycans, chick embryo skin fibroblast, conditioned medium, interleukin-bioactivity*

Introduction

ECM macromolecules are known to play key roles during normal cell life (for example, during development and aging), by regulating primary cell processes such as migration, adhesion, proliferation and differentiation (Bissel *et al.*, 1982; Ekblom *et al.*, 1986; Yoneda *et al.*, 1988). All of these activities are required for the building and maintenance of the specific architecture of any tissue. How the ECM regulates these processes is still largely unknown.

In the primary organ undergoing formation, specific classes of ECM glycosaminoglycans, proteoglycans and collagens accumulate in well-defined ratios and spatial distributions and are subjected to strict developmental regulation (Carinci *et al.*, 1981). In connective tissue, fibroblasts proliferate, synthesize and secrete ECM macromolecules and control the ECM composition depending upon developmental stage. These macromolecules surround the cells and influence both their secretory activity and proliferative ability (Evangelisti *et al.*, 1989; Bodo *et al.*, 1993). Chick embryo skin progressively accumulates HA and DS in respect to CS in the dermis during the stratification and keratinization of its overlying epidermis, whereas CS and HA accumulate in the lung mesenchyme during bronchial branching (Pane *et al.*, 1974, 1980; Becchetti *et*

Abbreviations used in this paper: CM, conditioned medium from untreated fibroblasts; CS, chondroitin 4 and 6 sulfate; DS, dermatan sulfate; ECM, extracellular matrix; FCS, fetal calf serum; GAG, glycosaminoglycans; HA, hyaluronic acid; HS, heparan sulfate; IL, interleukin; IL-1-CM, or IL-6-CM, respectively, CM from IL-1- or IL-6 treated fibroblasts; TGF, transforming growth factor.

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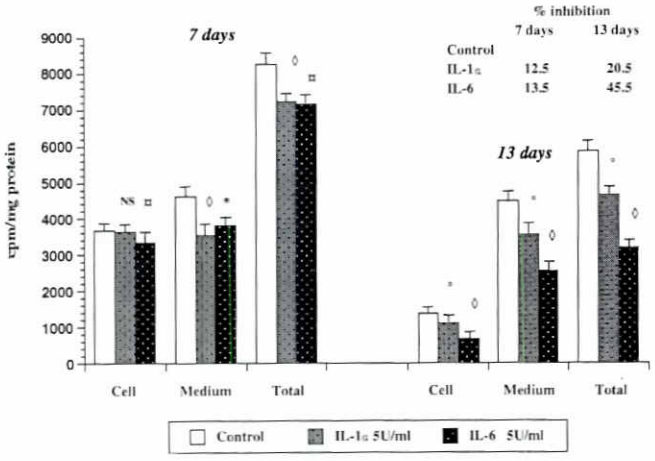

Fig. 1. ^{3}H-glucosamine incorporation into cellular and extracellular GAG in 7 and 13 incubation day fibroblasts maintained in medium 199±0.5% FCS with or without IL-1α or IL-6. *The values are expressed as cpm/mg protein and represent the mean±SD of two independent experiments each in quadruplicate. The differences were significant for P<0.02(*); P<0.05(¤); P<0.01(°); P<0.001(◊). NS= not significant.*

al., 1988). These molecules influence *in vitro* cell and tissue differentiation (Becchetti *et al.*, 1984; Carinci *et al.*, 1986). Thus, it is important to know both events and mechanisms that control ECM composition (Carinci *et al.*, 1978, 1991; Locci *et al.*, 1992).

Recently, growth factors have been shown to control the production of ECM components (Pierce *et al.*, 1991; Villaneuve *et al.*, 1991; Locci *et al.*, 1993). In particular, the role of cytokines in modifying connective tissue cell metabolism and in controlling fibroblast recruitment and ECM deposition during wound healing (Mauviel *et al.*, 1991) prompted us to explore the roles of IL-1 and IL-6 in the control of ECM turnover during embryogenesis. These two cytokines have many overlapping biological activities. IL-1 is a polypeptide produced by a wide variety of cells, such as monocytes, fibroblasts, B lymphocytes, natural killer (NK) cells, astrocytes, vascular endothelial cells, smooth muscle cells, thymic epithelial cells, epidermal cells and some T-lymphocyte cell lines (Luger and Oppenheim, 1983; Oppenheim *et al.*, 1986; Canalis *et al.*, 1987). Two biochemically distinct IL-1 molecules have been cloned: IL-1α and IL-1ß (March *et al.*, 1985; Qwarnstrom *et al.*, 1988). In recent studies, the action of IL-1 was shown to be mediated through IL-1 receptors of which at least two distinct forms (1 and 2) are known to exist (McMahan *et al.*, 1991; Kawaguchi *et al.*, 1993). The chicken IL-1 receptor has been particularly well-characterized (Guida *et al.*, 1992). Among the responses elicited by IL-1 are fibroblast proliferation (Schmidt *et al.*, 1982; Raines *et al.*, 1989), ECM degradation (Morris *et al.*, 1992), bone resorption (Thompson *et al.*, 1986), activation of T and B lymphocytes (Miossec *et al.*, 1984) and modifications of cytoskeleton and collagen synthesis (Bodo *et al.*, 1992a,b).

IL-6, whose receptor has been cloned (Savino *et al.*, 1993; Suzuki *et al.*, 1993), promotes B-cell differentiation, in particular immunoglobulin secretion, and is a co-factor for T-cell proliferation and differentiation by inducing IL-2 and IL-2 receptors (Foxwell *et al.*, 1992). *In vitro*, IL-6 has varying effects upon the growth of different tumor cell types. It stimulates the growth of plasmacytomas and is considered as an autocrine growth factor in multiple myeloma and Kaposi sarcoma (Revel, 1991). Conversely, IL-6 acts by inhibiting the growth of chronic lymphatic leukemia B-cells (Aderka *et al.*, 1992), myeloid leukemia cells (Gothelf *et al.*, 1991) and human breast carcinoma cell lines (Chen *et al.*, 1991). Thus, besides their immunoregulatory functions, interleukins can control the growth and differentiation of non-immune cells. We have already shown a key role of IL1 in bone metabolism (Bodo *et al.*, 1992b,c).

In this study, we were therefore interested in determining the influence of IL-1 and IL-6 upon synthesis and secretion of ECM glycosaminoglycans and collagen in chick embryo back skin fibroblasts cultured *in vitro* in two different developmental periods, 7 and 13 days, which correspond respectively to less differentiated and more differentiated stages of the skin (Carinci *et al.*, 1975). In fact in the back skin of 7 days *in vivo* the epithelium is bilayered and the mesenchyme initiates to condense to form feather primordia. At 13-14 days the feather buds are elongated, the interplumar epithelium is stratifying and the adjacent connective is loose.

In a parallel set of experiments we determined also whether or not chick embryo skin fibroblasts spontaneously secreted bioactive interleukins in the CM, and if their secretion could be modulated by IL-1 or IL-6 pretreatment of the cells. Secretion of interleukins into cell culture medium was quantitatively measured immunologically using IL specific ELISA kits, while cytokine bioactivity was evaluated by the ability of CM to stimulate the incorporation of ^{3}H-thymidine in target cells.

Results

Glycosaminoglycan biosynthesis

Fig. 1 shows the synthesis of both cellular and extracellular GAG in 7 and 13 day-old fibroblasts cultured in presence or absence of IL-1α and IL-6. At 7 days GAG accumulation was more evident than at 13 days, but at both developmental stages the treatment with the

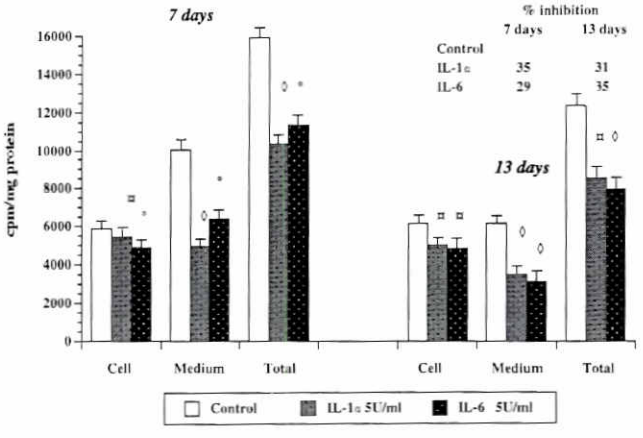

Fig. 2. $^{35}SO_4$ incorporation into cellular and extracellular GAG in 7 and 13 incubation day fibroblasts maintained in medium 199+0.5% FCS with or without IL-1α or IL-6. *The values are expressed as cpm/mg protein and represent the mean±SD of two independent experiments each in quadruplicate. The differences were significant for P<0.05(¤); P<0.01(°); P<0.001(◊).*

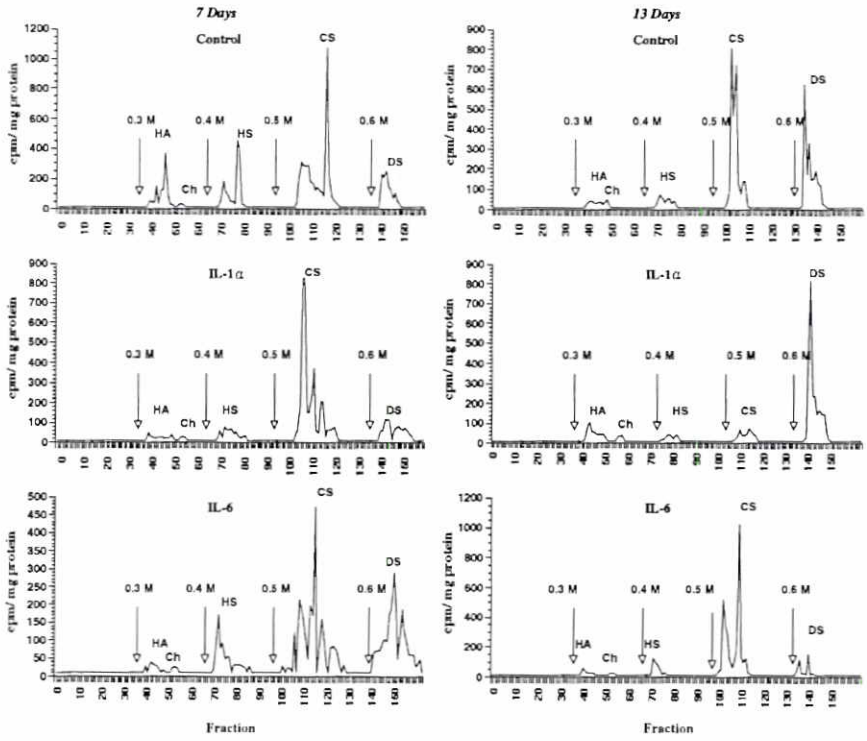

Fig. 3. Anion exchange elution profile of GAG synthesized by 7 and 13 day cultured fibroblasts (control, IL-1α or IL-6 treated).

interleukins caused decreases in synthesis, which were more marked for secreted GAG. The two interleukins provoked similar inhibitory effects on total GAG synthesis in 7 day-old fibroblast cultures, while in 13 day-old cells IL-6 was found to be more inhibitory than IL-1α (see insert in Fig. 1).

As for the synthesis and secretion of sulfated GAG (Fig. 2), it was found that treatment with both interleukins led to decreases in GAG levels in 7 and 13 day-old fibroblast cultures, which were more evident for secreted GAG. IL-1α was the more potent inhibitor in 7 day-old cultures and IL-6 in 13 day-old cells. Chromatographic analyses of CM samples permitted identification of individual GAG classes (Fig. 3 and Table 1). In 7 day-old fibroblasts, both interleukins significantly reduced values of HA and HS. Instead, DS value increased most notably upon IL-6 treatment, as did amounts of CS upon the administration of IL-1α. GAG profiles were different in 13 day-old treated fibroblasts. Here, IL-1 induced an increase of HA and DS, while IL-6 augmented HS and CS, and reduced DS.

Collagen biosynthesis

Collagen synthesis was diminished upon IL-1α and IL-6 treatment of cells (Fig. 4). This inhibition was evident for both secreted and cellular collagen. The relative amounts and distributions of collagen types in cell media were determined by gel electrophoresis and fluorography (Fig. 5). Cultured fibroblasts synthesized predominantly type I collagen, as shown by the position of the high molecular weight α1 and α2 bands. The polypeptides corresponding to the bands were susceptible to collagenase digestion (data not shown). Densitometric tracing of the autoradiograms was performed in order to determine the ratio of α2 to α1 chains (Table 2). After treatment of 7 day-old fibroblasts with both interleukins, the α2:α1 ratio increased. In 13 day-old fibroblast cultures, IL-1α did not induce a change in the ratio, while IL-6 caused a decrease.

Cytokine levels

ELISA analysis of CM (Table 3) demonstrated that both IL-1 and IL-6 are spontaneously produced by chick embryo skin fibroblasts, with highest concentrations found in the CM from 13 day-old fibroblasts. IL-6 was produced at much higher levels than IL-1, and of the two IL-1 isoforms, IL-1ß was found to be predominant. Pretreatment of 7 day-old fibroblasts with either IL-1α or IL-6 caused an increase in secretion of IL-1ß and IL-6. Levels of IL-1α

TABLE 1

PERCENTAGE OF ^{3}H-GLUCOSAMINE INCORPORATION INTO GAG CLASSES IN THE MEDIUM SECRETED BY 7 AND 13 DAY-OLD FIBROBLASTS MAINTAINED IN 199+0.5% FCS FOR 48 h WITH OR WITHOUT IL-1α or IL-6

	Medium					
	Total	Ch	HA	HS	CS	DS
7 days						
Control	4,605±708*	0.5**	14.7	19.5	52.5	12.8
IL-1α 5 U/ml	3,554±296	1	6.1	12.2	64.4	16.3
IL-6 5 U/ml	3,818±547	1	5.5	14.5	40.2	38.8
13 days						
Control	4,476±373*	0.53**	7.1	7.5	50.2	34.7
IL-1α 5 U/ml	3,542±467	0.59	11.5	6.5	10.3	71.2
IL-6 5 U/ml	2,523±252	0	7.1	12.8	62.4	17.5

*Total value of radioactivity (cpm/mg protein)±SD. ** % secretion in the medium. Ch, chondroitin; HA, hyaluronic acid; HS, heparan sulfate; CS, chondroitin-4 and 6-sulfate; DS, dermatan sulfate.

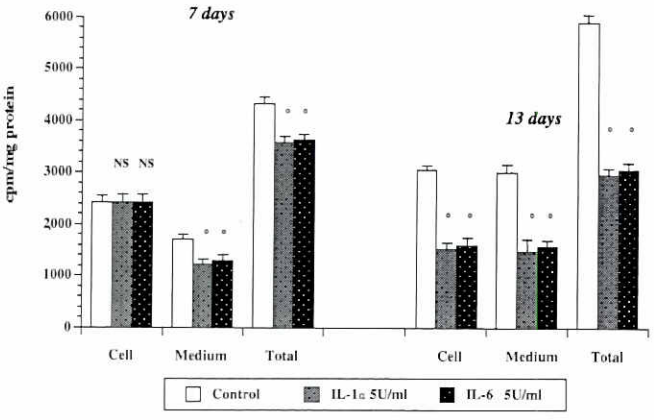

Fig. 4. Effect of IL-1α and IL-6 on collagen synthesis by 7 and 13 day-old chick embryo fibroblasts maintained in medium 199±0.5% FCS with or without IL-1α or IL-6. *The values are expressed as cmp/mg protein and represent the mean± SD of two independent expeiments each in quadruplicate. The differences were significant for P<0.01(°). NS= not significant.*

were significantly increased only by pretreatment of cells with IL-6 at lower dose. In the CM of 13 day-old fibroblasts, IL-1α and IL-6 levels were reduced following pretreatment of cells with both cytokines, while IL-1ß level was increased following administration of IL-6 at the higher dose.

Cytokine bioactivities

Fig. 6 shows IL-1 bioactivities in the CM of fibroblasts under various treatment conditions, as measured by stimulation of thymidine incorporation in mouse thymocytes. 7 day- and 13 day-CM increased ^{3}H-thymidine incorporation with respect to controls, and therefore contained IL-1 in a biologically active form. IL-1 activity was higher at 7 days than at 13 days. IL-1-CM failed to enhance IL-1 activity in respect to CM. In contrast, a dose-dependent increase in IL-1 biological activity was seen in the IL-6-CM. This increase, although seen in 7 day- and 13 day-CM, was more marked in 7 day-CM.

Results regarding IL-6 bioactivities are shown in Fig. 7. Both 7 and 13 day chick embryo fibroblasts secreted bioactive IL-6, with no obvious differences in concentration between the two developmental stages. Neither IL-1-CM nor IL-6-CM enhanced IL-6 activities with respect to those present in the CM.

Discussion

In the present study, we examined the effects *in vitro* of interleukin 1 and interleukin 6 upon the accumulation of ECM macromolecules by chick embryo skin fibroblasts at two different developmental stages and the ability of these cells, treated or not with cytokine, to release IL-1 and IL-6.

Our results show that both interleukins tested are capable of altering the neosynthesis and secretion of GAG and collagen. In particular, the cytokines diminished the accumulation of both tritiated and sulfated GAG, with the most marked inhibition seen upon IL-6 treatment in 13 day-old fibroblast cultures. Interleukin treatment modulated the secretion of specific GAG classes, which differed with respect to developmental stage. For example, at 7 days IL-6 increases DS, at 13 days IL-1 strongly decreases CS. The expression of collagen, most notably type I, was also down-regulated by the cytokines, with the greatest diminution seen in 13 day-old fibroblast cultures, treated with either interleukin. The ratio of α2 to α1 collagen chains also varied according to treatment. Reduced collagen levels induced by IL-1 and IL-6 might be explained by reduced synthesis of collagen itself, or if there were an IL-induced increase in collagenase expression, as has been hypothesized by others (Van Der Zu *et al.*, 1993).

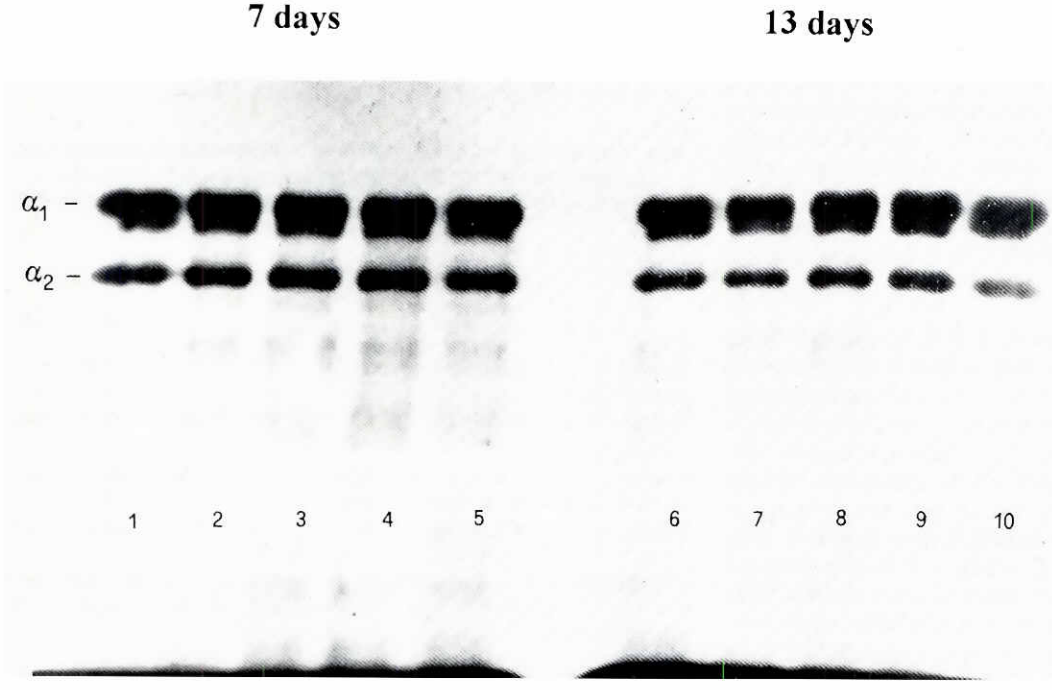

Fig. 5. Confluent cultures of 7 and 13 day chick embryo skin fibroblasts were treated with or without IL-1α or IL-6 for 48 h and labeled with ^{3}H-proline for 24 h. *The extracted collagen from the medium was electrophoresed on a 5% SDS-polyacrylamide gel and subjected to fluorography. The positions of α_1 and α_2 type I collagen chains are indicated.* **(Lanes 1,6)** *Controls.* **(Lanes 2,7)** *Cells treated with 1 U/ml IL-1α.* **(Lanes 3,8)** *Cells treated with 2.5 U/ml IL-1α.* **(Lanes 4,9)** *Cells treated with 1 U/ml IL-1α.* **(Lanes 3,8)** *Cells teated with 2.5 U/ml IL-1α.* **(Lanes 4,9)** *Cells treated with 1 U/ml IL-6.* **(Lanes 5,10)** *Cells treated with 2.5 U/ml IL-6.*

TABLE 2

DENSITOMETRIC ANALYSES AFTER FLUOROGRAPHY OF ^{3}H-COLLAGEN SECRETED INTO THE MEDIUM FROM CHICK EMBRYO FIBROBLASTS GROWN IN PRESENCE OR ABSENCE OF IL-1α OR IL-6

	7 days	13 days
Control	0.41	0.41
IL-1α 1 U/ml	0.52	0.39
IL-1α 2.5 U/ml	0.64	0.41
IL-6 1 U/ml	0.71	0.37
IL-6 2.5 U/ml	0.75	0.29

Values are the ratio of α_2 chain to α_1 chain.

Our results demonstrate the capacity of the cytokines to modulate the composition of the ECM, thereby lending support to the theory that ECM-cytokine interactions play an important role *in vivo*. In fact, GAG can capture and bind cytokines, thereby sequestering them at the cell membrane and tightly controlling their distribution (Rouslahty and Yamaguchi, 1991). The observed changes in GAG and collagen accumulation, induced by cytokine treatment in different developmental stages, confirm the theory that monokines are powerful regulators of developmental processes (Klasing and Johnstone, 1991) and morphogenesis.

The secretion of citokines undergoes an increase between 7 and 13 days of development, which could be related to the alterations in GAG accumulation which are seen during this period. Thus, chick embryo fibroblasts could control their own GAG synthesis through secretion of autocrine cytokine activities. The result that IL1 induces the secretion of IL-1ß and IL-6 has been observed by others using different cell systems (Warner *et al.*, 1987; Sironi *et al.*, 1989; Gadient *et al.*, 1990; Guerne *et al.*, 1990; Feghali *et al.*, 1992; Lacey *et al.*, 1993).

In addition, we observed that there is not always a correlation between the concentrations of cytokines present in tissue culture supernatants and the biological activities of these CM. In several instances, cytokine pretreatment of fibroblast cultures caused increased interleukin secretion, without concomitant increases in bioactivity. Additionally, whereas IL-1ß secretion is higher in 13 day-old fibroblast CM, supernatants from 7 day-old cultured fibroblasts exhibited more bioactivity. This different bioactivity could be explained by a specific ability of accumulated ECM components (as above reported) to interfere with the cytokine. This is, for example, the behavior of HA in respect to TGF; our unpublished data showed that HA interacts with TGFß, inhibiting its activity, but not with TGFα.

On the whole, our data are in agreement with what has been reported in other cell systems. IL-1 inhibits the synthesis of proteoglycans in cartilage cultures (Benton and Tyler, 1988; Venn *et al.*, 1990). Synthesis of collagen is stimulated during the proliferation of guinea-pig fibroblasts (Wahl *et al.*, 1978) and synovial cells in the presence of factors released from PHA-activated lymphocytes in serum-free medium (Parrot *et al.*, 1982). Others have shown that an IL-1-like factor provoked a reduction in collagen synthesis in fibroblasts maintained in 1% FCS (Jimenez *et al.*, 1979), in human chondrocytes (Gowen *et al.*, 1984; Pujol *et al.*, 1984) and in human dermal fibroblasts (Mauviel *et al.*, 1991). Differences in serum concentration and/or cell type may explain the apparently contrasting results of these studies. As yet, we do not know the molecular mechanisms by which the cytokines exert their effects upon embryonic cells. There have been reports that IL-1 signalling might be linked to activation of a G protein or elevations in cAMP levels or the action of protein kinase A or the activation of the transcription factor NFB (Mizel, 1990). Little is known of IL-6 signal transduction, although it has been shown that IL-6 induces tyrosine phosphorylation in gp130, an auxiliary signalling molecule (Taga *et al.*, 1990; Murakami *et al.*, 1991; Nishio *et al.*, 1993).

The effects of IL-1 and IL-6 upon the expression of ECM components may be direct or indirect, mediated by the induction of other growth factors, as has already been shown for IL-1 in other cell systems (Raines *et al.*, 1989; Arend, 1991). The effects of

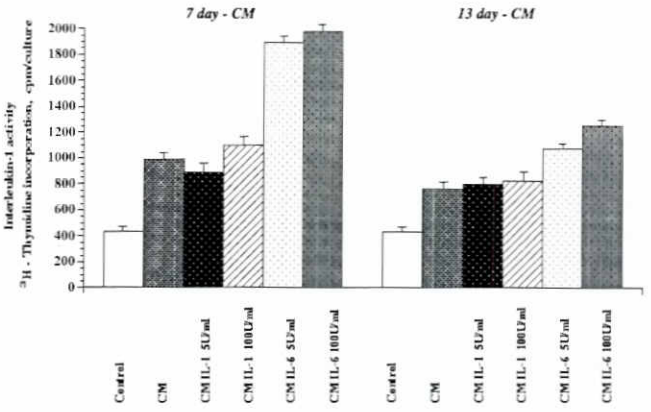

Fig. 6. IL-1 activities in conditioned media of untreated (CM) and IL-1 or IL-6 treated cells (IL-1 CM; IL-6 CM). *7 and 13 day chick embryo fibroblasts were cultured for 48 h in 5% FCS in medium 199 with or without IL-1α or IL-6 (5-100 U/ml). All cultures were then incubated for an additional 24 h in serum- and cytokine-free medium 199. Supernatants were harvested and tested for IL-1 activity, expressed as ^{3}H-thymidine incorporation in thymocytes. All differences from controls are significant for P<0.001.*

TABLE 3

CYTOKINE LEVELS IN CONDITIONED MEDIA OF UNTREATED IL-1α OR IL-6 TREATED CELLS

	Cytokine levels (pg/well)					
	7 day-CM			13 day-CM		
	IL-1α	IL-1ß	IL-6	IL-1α	IL-1ß	IL-6
CM	3.03	52	485	22.04	85	1,326
IL-1α CM 5 U/ml	2.08	93	673	15.03	59	1,102
IL-1α CM 100 U/ml	2.06	60	830	18.10	47	674
IL-6 CM 5 U/ml	25.10	147	1,558	9.12	72	1,164
IL-1α CM 100 U/ml	3.03	192	1,348	9.07	166	880

7 and 13 day chick embryo fibroblasts were maintained for 48 h in medium 199±5% FCS with or without IL-1α or IL-6 (5-100 U/ml) followed by 24 h in serum- and cytokine-free medium. Supernatants from untreated cultures (conditioned medium, CM) and cytokine-treated cultures (IL-1 CM or IL-6 CM) were harvested and the levels of IL-1α, IL-1ß and IL-6 measured using Elisa kits

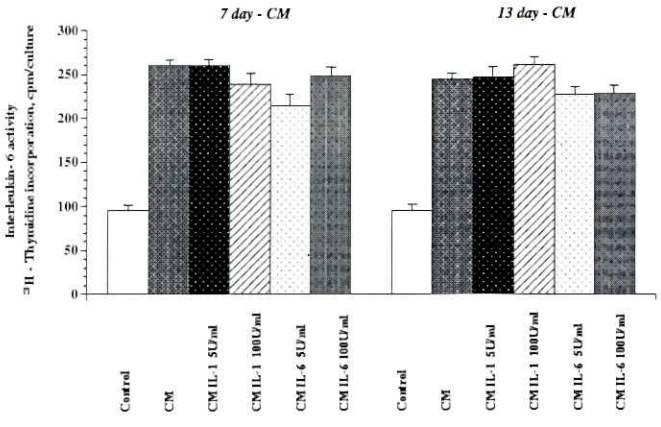

Fig. 7. IL-1 activities in conditioned media of untreated (CM) and IL-1 or IL-6 treated cells (IL-1 CM; IL-6 CM). *7 and 13 day chick embryo fibroblasts were cultured for 48 h in 5% FCS in medium 199 with or without IL-1α or IL-6 (5-100 U/ml). All cultures were then incubated for an additional 24 h in serum- and cytokine-free medium 199. Supernatants were harvested and tested for IL-6 activity, expressed as ^{3}H-thymidine incorporation in 7TD1 hybridoma cells. All differences from controls are significant for P<0.001.*

exogenous cytokines may be mediated through the release of IL-1 and IL-6 as autocrine growth factors. It remains to be shown whether the cytokines may induce other regulator peptides. In fact, it has been proposed that IL-1 effects may be mediated by TGFß, as this factor is produced by IL-1 treated cells (Danforth and Sgagias, 1993). TGF-ß is known to exert opposite effects in respect to IL-1 in some systems (Bry *et al.*, 1993; Redini *et al.*, 1993), to inhibit the expression of IL-1 receptors (Dubois *et al.*, 1990; Harvey *et al.*, 1991) and to induce the production of IL-1 receptor antagonists (Turner *et al.*, 1991). It has been pointed out that many of the actions of IL-1 and IL-6 are shared by TGF-ß, which may be because IL-1, but not IL-6, promotes TGF-ß production (Danforth and Sgagias, 1993). In the chick embryo fibroblast system, TGF-ß production is maximal in 13 day fibroblast cultures (Locci *et al.*, 1993). It is possible that pretreatment of the cells with cytokines may modulate the production of TGF-ß or some other factor, thereby masking or amplifying the effects of the secreted cytokines.

Given that the cytokines transmit their signals through high affinity interactions with specific cell surface receptors and that synthesis of ECM macromolecules varies according to developmental stage, experiments are now under way to verify the expression of IL receptors at the various stages.

Materials and Methods

Cell cultures

Hubbard fertilized eggs provided by the Ci.C.Zoo-Zootecnica incubator company (Perugia, Italy) were incubated at 38°C at a relative humidity of 60%. Back skin fragments were carefully removed under sterile conditions from 7 and 13 day-old chick embryos staged according to Hamilton (1952), cut into small pieces and dissociated in 0.25% trypsin (1/250; DIFCO Laboratories, Detroit, MI, USA), in Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution at room temperature for 30 min. The dissociated cells were filtered through a nylon mesh, centrifuged (10 min at 350 g), then washed with phosphate-buffered saline (PBS) (pH 7.4) and suspended in medium 199 (GIBCO, Grand Island, NY, USA) plus 10% fetal calf serum (FCS) (GIBCO). Cell suspension ($1x10^6$ cells/ml) was plated and maintained in a humidity-saturated atmosphere (5% CO_2, 37°C) for 24 h. Cells, obtained as previously described, were nearly exclusively fibroblasts. Media were then exchanged for either medium 199 plus 0.5% FCS or medium with the addition of recombinant human IL-1α or IL-6 (Boehringer Mannheim Italia S.p.A, Italy) at a concentration of 2.5-5 U/ml and the cultures were maintained for 48 h. Cell viability was measured by trypan blue exclusion assay (Patterson, 1979). Cells were counted using a Burker's chamber.

Newly synthesized GAG isolation and identification

Five ml of cell suspensions ($1x10^6$ cells/ml) were plated in culture flasks for 48 h in medium 199 plus 0.5% FCS supplemented with IL-1α or IL-6 (5 U/ml) and labelled with 5 mCi/ml of ^{3}H-glucosamine hydrochloride (NEN Du Pont de Nemours, RFG; s.a. 29 Ci/mmol) or 5 µCi/ml of $^{35}SO_4$ (NEN; s.a. 1,200/1,400 Ci/mmol) for the last 24 h. At the end of the incubation, cells and media were recovered separately. Cells were scraped in 1 ml ice-cold 0.1 M Tris-HCl and 1.5 mM $CaCl_2$ (pH 8.2) and lysed by sonicating with six 10 sec bursts using a B15P model Branson sonicator. Media were dialyzed, lyophilized and dissolved in the above buffer. Lysates and media were boiled for 5 min and then digested for 3 days at 37°C with 1 mg/ml predigested (for 30 min at 37°C) protease type XIV (Sigma Chemical Co., St. Louis, MO, USA) in the presence of 1% toluene. Protease was added fresh daily. Proteins in the digested samples were precipitated with 10% TCA, pelleted by centrifugation and discarded. GAG was precipitated from recovered supernatants with 3 volumes of 5% potassium acetate in absolute ethanol in the presence of 100 µg of GAG carrier and pelleted by centrifugation (for 20 min at 12,000g). Pellets were solubilized in 10 mM Tris-HCl (pH 7.2) and chromatographed on a DE-52 cellulose anion exchange column (0.8x4 cm; Whatman) equilibrated with 10 mM Tris-HCl (pH 7.2). Elution was carried out at room temperature with a NaCl gradient. Fractions of 0.6 ml were collected; 0.2 ml aliquots were mixed with 10 ml of Aquasol-2 (NEN) and counted in an LKB scintillation counter. Recovery of radioactivity was 85-90%. Column fractions corresponding to each peak were pooled, dialyzed and lyophilized. Individual GAG classes were identified by elution profile and enzymatic susceptibility to Streptomyces hyaluronate lyase and chondroitin ABC lyase (Seikagaku Kogyo Co., Japan) (Conrad *et al.*, 1977). Results are expressed as cpm/mg of protein.

Collagen synthesis

Fibroblasts were cultured for 48 h in medium 199 plus 0.5% FCS, 50 µg/ml L-ascorbic acid and 50 µg/ml ß-aminoproprionitrile fumarate with and without IL-1α or IL-6 (2.5-5 U/ml) in the presence of 1 µCi/ml of ^{3}H-labelled proline (s.a. 29 Ci/mmol; Amersham International, England) in the last 24 h. Collagen was extracted as described elsewhere (Webster and Harvey, 1979). Briefly, cell monolayers and media were recovered separately and treated with 100 µl of cold acetic acid (0.5 M); 250 µl NaCl in 0.5 M acetic acid was added and collagen pelleted by centrifugation at 4000 g for 20 min using 200 µg/ml rat skin collagen as a carrier. Precipitates were dissolved in 250 µl of 0.5 M acetic acid and transferred to vial inserts containing 2 ml scintillation fluid. Results are expressed as dpm/10^6 cells. The specificity of the assay was verified by monitoring the susceptibility of precipitates to purified bacterial collagenase (Calbiochem-Novabiochem Co.,CA, USA).

Analysis of collagen types

Aliquots of culture media containing $0.5\text{-}2x10^4$ dpm were lyophilized and redissolved in 100 µl of 0.5 M acetic acid adjusted to pH 2.2 with HCl. Samples were digested with 50 µg pepsin (Sigma Chemical Co., MO, USA) dissolved in the same solution for 16 h at 4°C. The solution was immediately lyophilized and subsequently analyzed by SDS-PAGE on 5% cross-linked gels. Collagen a chains, corresponding to type I collagen, were visualized by fluorography and quantified via densitometric scanning using the Quanti Scan program.

Preparation of conditioned media (CM)

After cytokine treatment, cells underwent prolonged washing for 6 h followed by a 24 h incubation in 199 medium alone. Tissue culture supernatants were harvested from untreated cells (CM) and cytokine-treated cells (IL-1-CM or IL-6-CM), and centrifuged for 10 min at 2,000g in

the presence of protease inhibitors (1 mM phenylmethylsulfonylfluoride) to remove cells and debris. Supernatants were then dialyzed against water for 72 h, lyophilized and assayed for IL-1 or IL-6 bioactivity.

Thymocyte proliferation assay for IL-1 activity

Aliquots of CM, IL-1-CM and IL-6-CM were assayed for IL-1 activity by their ability to enhance proliferation of mouse thymocytes as described previously (Warner *et al.*, 1987). Briefly, $1.5x10^6$ thymocytes isolated from 6 week old mice were cultured in 0.2 ml RPMI 1640 medium containing 10% FCS (control) or aliquots of CM, IL-1-CM or IL-6-CM in microtitre culture plates (Falcon 3072, Becton Dickinson Labware, NJ, USA) at 37°C for 3 days in a 5% CO_2 humidified atmosphere. Cells were pulsed with ^{3}H-thymidine (5 μCi/well) (s.a. 29 Ci/mmol; Amersham) for the final 24 h of culture and then harvested using an automated cell harvester. Radioactivity was counted in a liquid scintillation counter. Results are expressed as the mean cpm±SD of quadruplicate determinations.

Proliferation assay for IL-6 activity

Assay was performed as described elsewhere (Van Damme *et al.*, 1987). Briefly, IL-6-dependent mouse 7TD1 hybridoma cells were cultured at a density of 3,000 cells/well in 0.2 ml of RPMI 1640 plus 10% FCS (control) plus aliquots of CM, IL-1-CM or IL-6-CM in microtitre culture plates at 37°C for 3 days in a 5% CO2 humidified atmosphere. Cultures were pulsed with ^{3}H-thymidine (0.5 μCi/well) during the final 16 h of incubation. Cells were harvested using an automated cell harvester and radioactivity counted in a liquid scintillation counter. Results are expressed as mean cpm±SD of quadruplicate determinations.

ELISAs for IL-1α, IL-1ß and IL-6

Levels of the cytokines in CM, IL-1-CM and IL-6-CM were determined using specific ELISA kits for IL-1α (sensitivity 0.04-0.2 pg/ml; Biotrak, Amersham International, England) IL-1ß and IL-6 (sensitivity 30-60 pg/ml and 100 pg/ml respectively; Biochrom KG, Germany). Assays were performed according to manufacturers instructions. All samples were assayed in triplicate. Results are expressed as pg/well.

Statistical analysis

Statistical analysis was performed using Student's *t* test.

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