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Original Article

EGF and TGFα influence *in vitro* lung development by the induction of matrix-degrading metalloproteinases

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ABSTRACT **Remodeling of the extracellular matrix by matrix-degrading metalloproteinases (MMPs) has been implicated in the early morphogenesis of branched organs. Growth factors such as EGF and TGFα are known to regulate the expression of MMPs in a variety of systems. We therefore examined the effects of EGF, TGFα, and collagenase upon *in vitro* branching of the embryonic lung. Lung rudiments from 11.5 day *post coitum* mice underwent extensive growth and repetitive branching during a 3-day period in organ culture. Lungs treated with EGF or TGFα were larger than controls, yet displayed fewer branches along with markedly dilated end buds which lacked clefts, indicating that these growth factors inhibit normal lung branching. Addition of purified mammalian collagenase to lung cultures similarly inhibited epithelial branching and produced end bud enlargement. In addition, gelatin-substrate enzymography of the conditioned medium from EGF- and TGFα-treated lungs revealed a marked induction of a metalloproteinase activity which most likely corresponds to the 72kDa type IV collagenase/gelatinase which degrades basement membrane collagens. Lungs maintained in the presence of both TGFα and TIMP, a specific inhibitor of MMPs, branched repeatedly and displayed normal, narrow end buds as seen with controls, suggesting that TIMP is capable of preventing or reversing the observed growth factor mediated effects upon lung branching. Taken together, these results provide evidence that the growth factors EGF and TGFα guide lung development, at least in part, by inducing the expression of matrix-degrading metalloproteinases.**

KEY WORDS: *lung development, metalloproteinases, EGF, TIMP, gelatinase*

Introduction

The development of branched organs, including the lung, is dependent upon instructive interactions between mesenchyme and branching epithelium (Wessells, 1977). Although the molecular signals guiding branching morphogenesis are largely uncharacterized, considerable evidence implicates the extracellular matrix (ECM) as a key coordinator of morphogenetic events. For example, studies by Alescio (1973) and Spooner and Faubion (1980) have demonstrated that inhibitors of procollagen biosynthesis and secretion perturb *in vitro* branching morphogenesis of the lung and salivary gland. In a more recent report, lung explants treated with anti-laminin antibodies displayed a marked inhibition of growth and branching and a distortion of the bronchial tree (Schuger *et al.*, 1990). Furthermore, Li *et al.* (1987) have shown that the ability of cultured mouse mammary epithelial cells to grow in a manner resembling normal glandular epithelium and to express the differentiated gene product ß-casein is highly dependent upon the molecular constituents and overall structure of the matrix upon which the cells are plated.

The matrix metalloproteinases are a family of extracellular matrix-degrading enzymes recognized for their role in malignant tumor cell invasion and metastasis, wound healing, and inflammatory diseases such as rheumatoid arthritis (Werb, 1988; Liotta and Stetler-Stevenson, 1990; Matrisian, 1990). Members of the MMP family are divided into three major subclasses based upon substrate specificity and sequence similarity. The prototypes of these classes are interstitial collagenase, type IV collagenase/gelatinase, and stromelysin (Matrisian, 1990). MMPs are inhibited by a specific tissue inhibitor of metalloproteinases (TIMP), a 28.5-kDa glycoprotein produced by many different cell types (Welgus and Stricklin, 1983; Murphy and Reynolds, 1985). This protein recognizes the active forms of stromelysin and collagenase, binds them with a 1:1 stoichiometry, and inhibits their enzymatic activity (Welgus *et al.*, 1979; Cawston *et al.*, 1983). Another TIMP, called TIMP-2, has been isolated as a complex with the 72-kDa type IV collagenase

Abbreviations used in this paper: MMP, matrix metalloproteinase; EGF, epidermrowth factor; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix; FGF, fibroblast growth factor; APMA, 4-aminophenylmercuric acetate.

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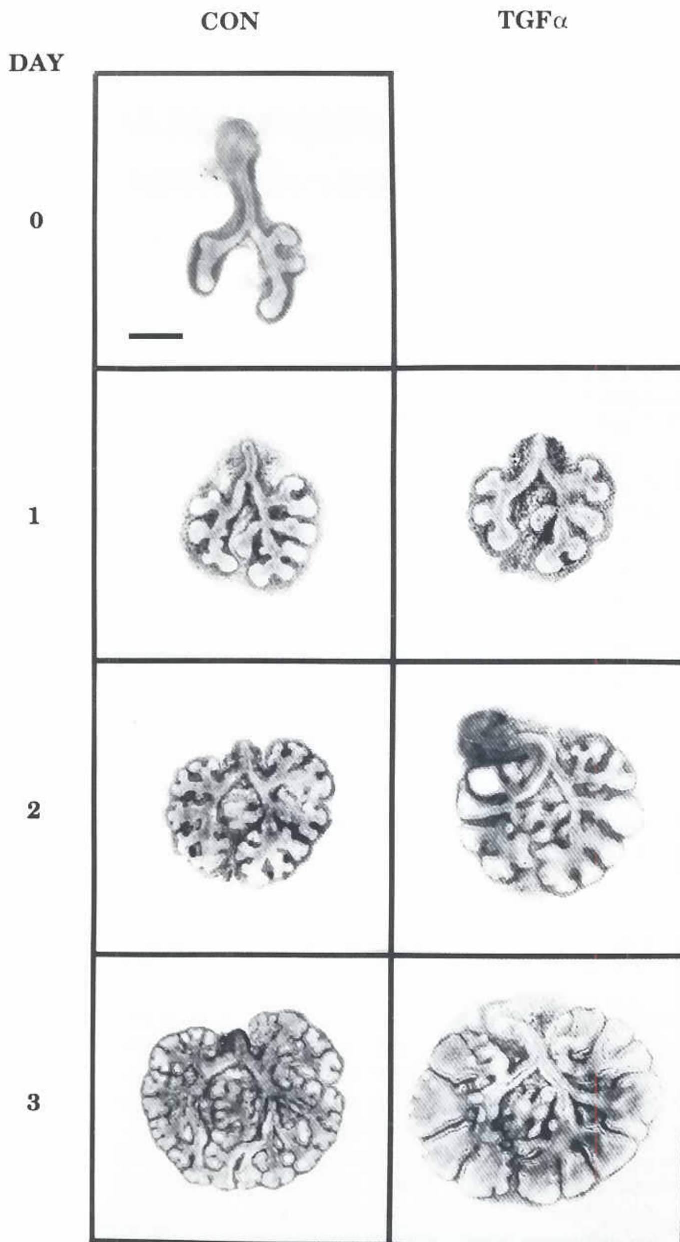

Fig. 1. Organ cultures of lung rudiments. *Control lung explants (CON) immediately after dissection and on days 1-3 of culture are shown compared to those treated with daily addition of 50 ng/ml TGFα (TGFα). Each picture represents a different lung and is representative of 8-10 lungs. Bar, 500 μm. All subsequent lung photographs are at the same magnification.*

(Goldberg *et al.*, 1989; Stetler-Stevenson *et al.*, 1989). The net metalloproteinase activity in a system is ultimately dependent upon the balance between the levels of activated proteases and their inhibitors, and the relative spatial localizations of the two.

Extracellular matrix remodeling by MMPs has previously been implicated in the early development of branched organs. For example, Wessells and Cohen (1968) have demonstrated that bacterial collagenases alter the *in vitro* morphogenesis of the embryonic lung and uretric bud. Similarly, bacterial collagenase treatment of the 12-day mouse submandibular gland profoundly inhibits cleft formation *in vivo*, whereas addition of a purified collagenase inhibitor markedly enhances epithelial cleft formation (Grobstein and Cohen, 1965; Nakanishi *et al.*, 1986). These studies indicate that a dynamic interplay between matrix degrading enzymes and their inhibitors is required for proper epithelial branching. Transcripts encoding several metalloproteinases have been detected in preimplantation embryos, a finding which is consistent with roles for these enzymes in blastocyst implantation (Brenner *et al.*, 1989). Moreover, functions for MMPs in numerous developmental processes characterized by extensive matrix remodeling have been proposed (for review see Matrisian and Hogan, 1990).

In all cell types examined thus far, expression of the MMPs is tightly regulated by numerous biological modifiers, including growth factors, cytokines, tumor promoters, and oncogenes (for review see Matrisian and Hogan, 1990). EGF and TGFα are two growth factors which can stimulate MMP expression in a variety of systems (see Chua *et al.*, 1985; Kerr *et al.*, 1988, for examples). EGF is widely known for its ability to induce proliferation in epithelial and other cell types (Carpenter and Cohen, 1979), an effect which is mediated by specific binding to the EGF receptor (Carpenter, 1987). The mature TGFα molecule shares 30% amino acid sequence similarity with EGF and binds to the same receptor (reviewed by Derynck, 1988). TGFα expression was originally thought to be restricted to neoplasia (Sporn and Todaro, 1980), but has since been found to include a wide range of normal cells and tissues (Derynck, 1988; Snedeker *et al.*, 1991).

During embryonic development, EGF and/or TGFα, and the EGF receptor are expressed in distinct temporal and tissue-specific patterns, implying crucial roles for these molecules in the development of many organs, including the lung (for reviews see Adamson, 1990, and Partanen, 1990). The majority of EGF-like activity isolated from murine embryos is not authentic EGF, but apparently TGFα (Nexo *et al.*, 1980). EGF is reported to be undetectable from 9 days of gestation through early neonatal development using blot hybridization techniques (Popliker *et al.*, 1987), while TGFα mRNA is expressed at high levels in mouse embryos at days 9-10 of gestation (Lee, *et al.*, 1985). Using the more sensitive methods of immunolocalization and *in situ* hybridization, however, Snead *et al.* (1989) detected expression of the EGF precursor protein in bronchial epithelial cells and of both the EGF protein and mRNA in the surrounding mesenchyme of developing murine lungs. Also, Warburton *et al.* (1989), used the polymerase chain reaction to identify expression of both EGF and TGFα mRNA by day-11 through day-17 mouse lung tissue. Binding of [^{125}I]EGF has localized the EGF receptor to both the mesenchyme and epithelium of developing lungs (Partanen and Thesleff, 1987). EGF stimulates proliferation of lung epithelium *in vitro* (Goldin and Opperman, 1980), and accelerates lung maturation in fetal rabbits (Catterton *et al.*, 1979) and lambs (Sundell *et al.*, 1980).

The incomplete understanding of how growth factors and other molecules coordinate epithelial branching, along with the known involvement of MMPs in matrix remodeling, prompted us to examine the roles that growth factor regulated proteases may play in lung morphogenesis. This study provides evidence that the EGF and TGFα regulation of lung morphogenesis in culture, and therefore possibly during *in vivo* development, involves the induction of matrix metalloproteinases.

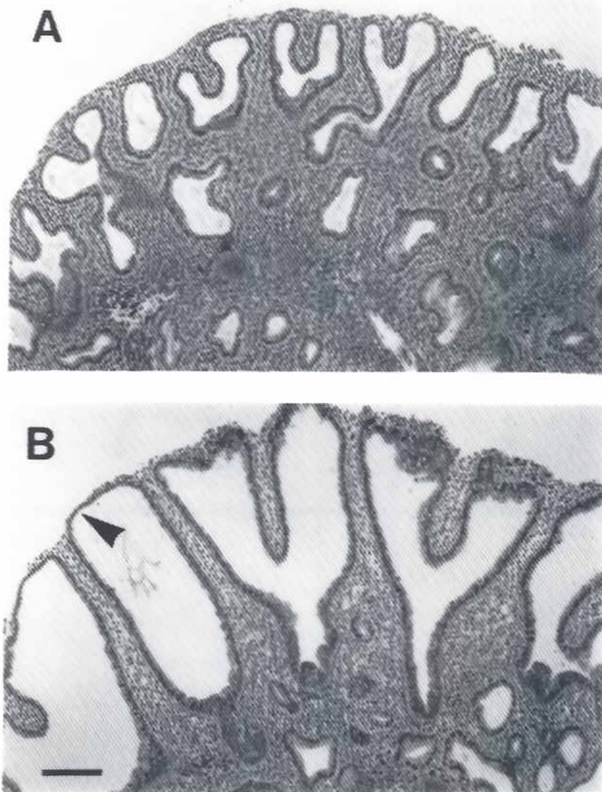

Fig. 2. Effects of TGFα on lung end bud morphology. *Longitudinal sections were taken through explants maintained in culture for 3 days with no growth factor addition (A), or in the presence of 50 ng/ml TGFα (B) and stained with hematoxylin and eosin. An arrowhead indicates a dilated end bud in which the epithelium extends beyond the boundary of the mesenchyme. Bar, 100 μm.*

Results

Lung rudiments of 11.5-day p.c. embryos have a simple morphology consisting of the right and left primary bronchi each with 2-3 secondary bronchi (Fig. 1, day 0). These rudiments underwent extensive growth and branching during the 3-day period in culture (Fig. 1, day 0-3). The daily addition of 50 ng/ml TGFα to the cultures had a marked effect upon the branching pattern of the epithelium, such that by day 2, TGFα-treated lungs exhibited fewer branches than controls. The most striking alteration was seen at the lung periphery where TGFα-treated organs exhibited a marked dilatation of epithelial end buds, a feature which became increasingly prominent during the culture period. The overall size of TGFα-treated lungs as estimated from the area of photographed rudiments was also somewhat increased on days 2 and 3 relative to controls (3.99±0.3 mm^2 as compared to 3.30±0.21 mm^2 for control rudiments measured on day 3).

Histological examination of control and TGFα-treated lungs after 3 days in culture revealed no obvious differences in the microarchitecture of the epithelium or the mesenchyme (Fig. 2). In TGFα-treated rudiments, however, the broadening of the distal regions of the branches was seen as large luminal spaces lined by well-organized, simple columnar epithelium. The most peripheral epithelium was occasionally observed to extend beyond the boundary of the mesenchyme, a characteristic not seen with control rudiments.

We next examined the dose response of branching inhibition by TGFα and compared the results to those seen with EGF (Fig. 3). Rudiments treated with 10 and 50 ng/ml of either EGF or TGFα were slightly larger than control rudiments, with the increase in size being roughly proportional to the growth factor concentration (see Figure legend for area measurements). The end buds of lungs maintained at 10 and 50 ng/ml of either growth factor were dilated to comparable levels. However, the branches in the central regions of lungs maintained at 10 ng/ml were rather crowded in appearance compared to the broader, more dispersed branches in the central regions of lungs maintained at 50 ng/ml. A slight inhibition of branching could be seen with EGF and TGFα concentrations as low as 2 ng/ml, and concentrations greater than 50 ng/ml up to 200 ng/ml of either growth factor caused changes similar to those seen at 50 ng/ml (data not shown). Although the precise levels of EGF or TGFα in developing mouse lungs has not been determined, the presence of approximately 80 ng/ml of EGF receptor-binding material in mouse serum suggests that these concentrations are physiologically relevant (Ladda *et al.*, 1979). At each concentration tested, morphological changes induced by EGF were indistinguishable from those caused by TGFα.

In previous studies, purified *Clostridial* collagenases have been found to strongly inhibit cleft formation of 12-day and 13-day submaxillary rudiments (Grobstein and Cohen, 1965; Nakanishi, *et al.*, 1986). These findings have suggested roles for endogenous MMPs in branching morphogenesis. We therefore tested the effects of bacterial collagenase upon lung morphogenesis in organ culture. Inhibition of lung branching by 1 μg/ml bacterial collagenase was evident by day 1 and persisted throughout the culture period (data not shown). Bacterial collagenases cleave all collagens at multiple sites, and certain preparations are known to contain additional proteolytic activities. We therefore extended this study by testing the effects of purified mammalian collagenase, which degrades interstitial collagens of type I, II, and III in a highly specific manner, upon lung rudiments. The epithelial branching pattern of collagenase-treated lungs was altered in a dose-dependent manner. At a collagenase concentration of 2 μg/ml, at least some peripheral lung segments were able to form clefts, and therefore showed evidence of recent branching, whereas other segments were dilated and unbranched. At collagenase concentrations of 3 μg/ml and above, no further branching of any segments beyond day 1 was noted. Instead, the lumina of existing branches became increasingly broadened on days 2 and 3, with the tips of the branches being particularly dilated.

In general, concentrations of mammalian collagenase less than 2 μg/ml produced less dramatic and temporally delayed branching effects relative to those seen with higher collagenase levels (data not shown). Concentrations of collagenase greater than 6 μg/ml frequently appeared to compromise lung viability and occasionally caused rudiments to detach from the nucleopore filters. In control experiments where the collagenase solvent and activator alone (Brij-35, trypsin, and trypsin inhibitor) were added to lungs, no alteration of branching was noted (compare lung 0 of Fig. 3 to control lungs of Figs. 1 and 5).

Because of the similarities of end bud structure seen following EGF, TGFα, and collagenase treatment, we investigated whether the

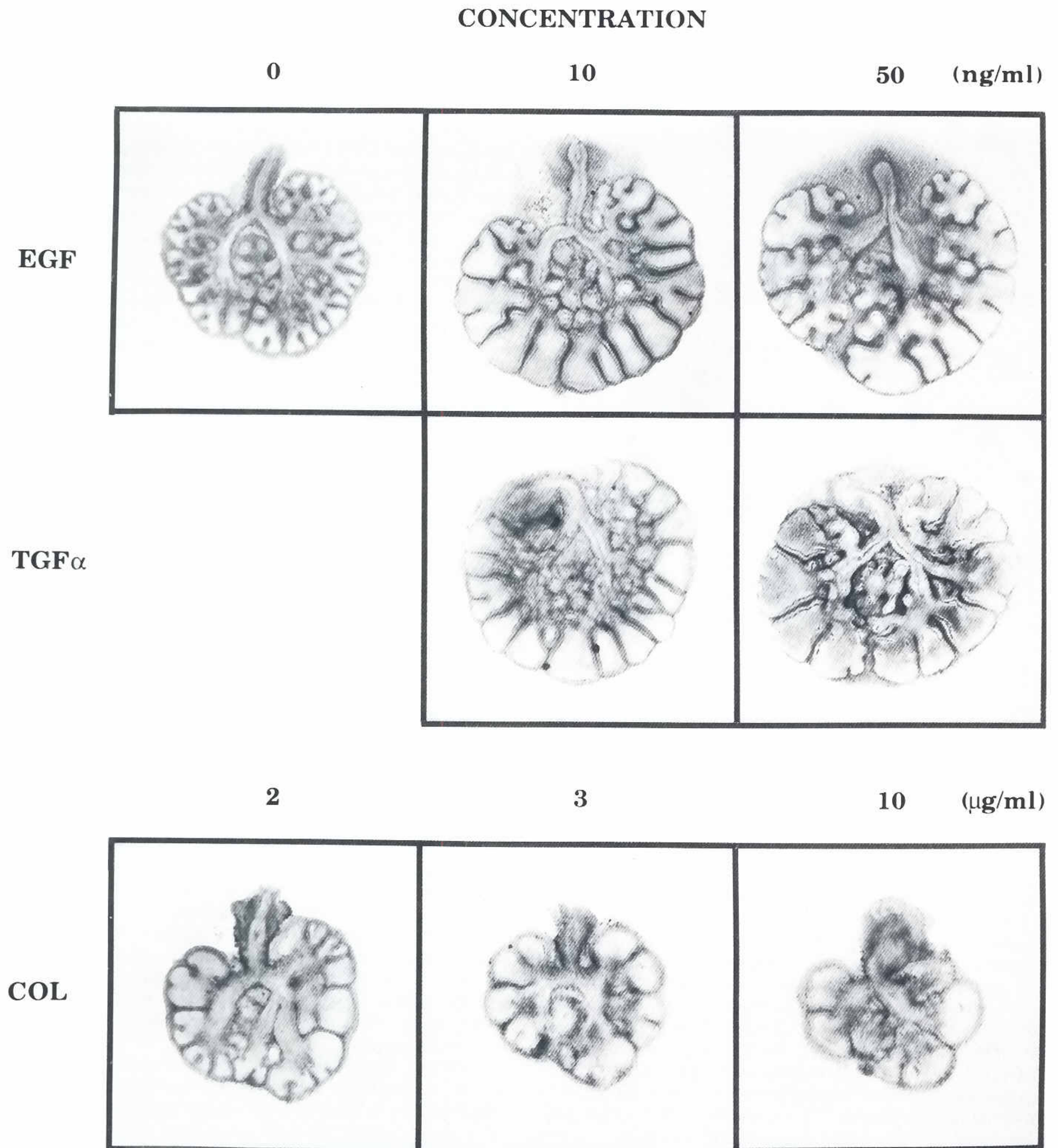

Fig. 3. Dose response of EGF, TGFα, and mammalian collagenase effects upon lung branching. *Lung rudiments were maintained for 3 days in the presence of the indicated concentrations of EGF, TGFα, and mammalian collagenase (COL). The control lung pictured (0) is representative of rudiments exposed to equivalent amounts of Brij-35, trypsin, and trypsin inhibitor as the collagenase treated lungs. Each lung is representative of 4-5 lungs per group. Some lungs shown are also included in other Figs.; they are presented here for comparison purposes. The sizes of the rudiments as determined by morphometric analysis (mean± S.D.) were: control, 3.30± 0.21 (n=4); 10 ng/ml EGF or TGFα, 3.57± 0.29 (n=5); 50 ng/ml EGF or TGFα 3.99± 0.30 (n=3); 2 μg/ml collagenase, 2.59± 0.13 (n=3).*

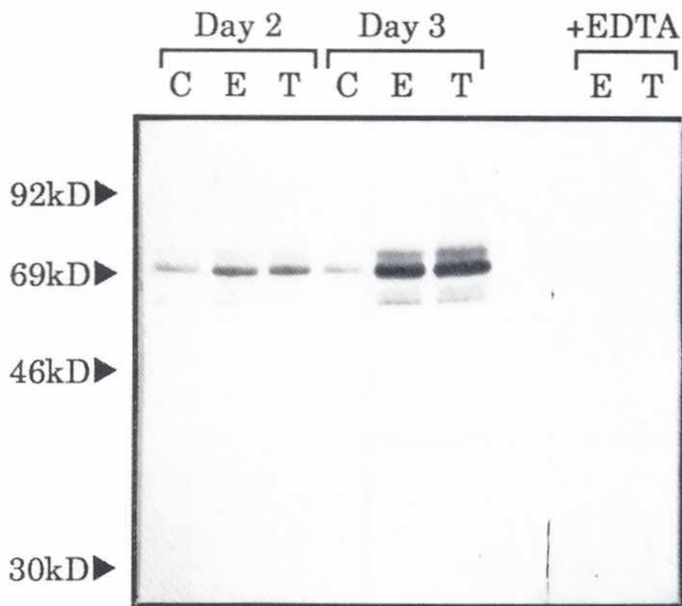

Fig. 4. Gelatin-substrate enzymography of medium conditioned by control, EGF and TGFα-treated lung rudiments. *Lungs were cultured for 3 days with or without (C) daily addition of 50 ng/ml EGF (E) or 50 ng/ml TGFα (T). Medium conditioned by 4 lungs per group from hours 24-48 (Day 2) and 48-72 (Day 3) of culture was collected and concentrated. Each lane represents the medium conditioned by approximately 0.6 lungs for 24 hours. Lanes from the zymogram were incubated in substrate buffer alone (first 6 lanes) or in buffer containing 10 mM EDTA (+EDTA). Samples shown in +EDTA lanes were identical to Day 3, E and T samples. Molecular weight markers are shown on the left. The zymogram is presented as a negative image for clarity.*

growth factor regulation of endogenous MMPs could be responsible for the effects of EGF and TGFα upon lung branching. First, we analyzed the relative levels of metalloproteinase activities in medium conditioned by control versus EGF and TGFα-treated lungs. Medium was collected daily, concentrated, and analyzed on polyacrylamide gels containing gelatin or casein. Gelatin substrate zymography revealed that the levels of a major gelatinolytic activity migrating at 72-kDa, and minor species at 78 and 63-kDa, were increased several fold in medium from lungs treated with 50 ng/ml EGF or 50ng/ml TGFα (Fig. 4). Zymographic analysis of medium conditioned from hours 0-24 of culture showed induction of gelatinolytic activities similar to that seen on day 2, although both control and induced levels were lower (data not shown). Incubation of duplicate electrophoresed samples in substrate buffer containing EDTA, a potent inhibitor of metalloproteinases, prevented the activity of all three gelatinolytic species (Fig. 4, lanes 7 and 8). Upon enzyme activation by the organomercurial APMA, the 72-kDa band partially shifted to a lower 67-kDa form (data not shown), consistent with it being the 72-kDa type IV collagenase. Casein substrate gels revealed no detectable caseinolytic activity present in any of the lung cultures.

The observed growth factor induction of endogenous metalloproteinases suggested that EGF and TGFα induction of MMPs may play a key role in the effects seen following growth factor addition. To further test this hypothesis, we added the specific MMP inhibitor TIMP to lung cultures, either with or without the coaddition of TGFα or EGF. Incubation with 50 μg/ml TIMP alone produced no obvious changes in morphology relative to controls by day 3 of culture (Fig. 5). Treatment with 10 ng/ml TGFα alone produced effects similar to those seen previously (see Fig. 3). Lungs treated with both TGFα and TIMP, however, acquired branching patterns resembling those of control explants. Peripheral lung segments of TGFα+TIMP-treated lungs in general were undilated and exhibited evidence of continued cleft formation and branching. In order to quantitate the effect of the different treatments upon branching of lung rudiments, we determined the branching density expressed as the number of end buds present per mm^2 of lung tissue as described in Materials and Methods (Table 1). This analysis revealed that control and TIMP-treated rudiments possess an average of 24.1 and 23.9 branches/mm^2, respectively. On the other hand, TGFα-treated lungs display an average of 15.2 branches/mm^2. Addition of TIMP+TGFα allows branching similar to control levels with an average of 25.8 branches/mm^2. These treatments had no significant effect on total lung area (Table 1). Substitution of EGF for TGFα in this experiment produced similar results. In cultures utilizing higher concentrations of EGF or TGFα (50 ng/ml), an equivalent amount of TIMP was slightly less effective at reversing the growth factor effects (data not shown).

Discussion

In this study, we have utilized an organ culture system to address the mechanisms by which TGFα and EGF control lung morphogenesis. TGFα and EGF treatment of 11.5-day lung rudiments inhibited branch formation and caused dilatation of epithelial end buds in a dose-dependent manner, an effect which was mimicked by treatment with collagenase. In addition, we demonstrated the growth factor induction of metalloproteinase activities, and finally, the ability of TIMP, a specific inhibitor of MMPs, to reverse the growth factor mediated effects upon lung branching. Together, these results strongly suggest that the observable effects of EGF and TGFα upon *in vitro* lung branching are mediated by an induction of metalloproteinase activities.

At this time we cannot positively identify the specific metalloproteinase(s) that mediates the growth factor effects on lung branching. The apparent molecular weights of the gelatinolytic polypeptides induced by EGF and TGFα (Fig. 4), their inhibition by EDTA, and their size shift with APMA suggest that the prominent 72-kDa species is the 72-kDa type IV collagenase/gelatinase (MMP-2), while the 62-kDa band most likely represents a lower MW form of the 72-kDa proenzyme (Overall, *et al.*, 1989). The human type IV collagenase/gelatinase preferentially degrades gelatins, type IV collagen, type V collagen, fibronectin, and type VII collagen, but does not cleave type I collagen or laminin (Collier *et al.*, 1988). The basement membrane-degrading properties of the 72-kDa enzyme have long been associated with tumor invasion and metastasis (for review see Liotta and Stetler-Stevenson, 1990).

The addition of the purified human interstitial collagenase to lung cultures produced effects similar to those seen following growth factor addition. This enzyme is known to specifically cleave all three chains of mature, triple-helical types I, II, and III collagen at a specific locus, but has also been shown to hydrolyze gelatins (Stricklin *et al.*, 1978) as well as several other substrates (Fields *et al.*, 1990, and references therein). Due to the relatively restricted substrate specificity of collagenase, we presume the effect of this

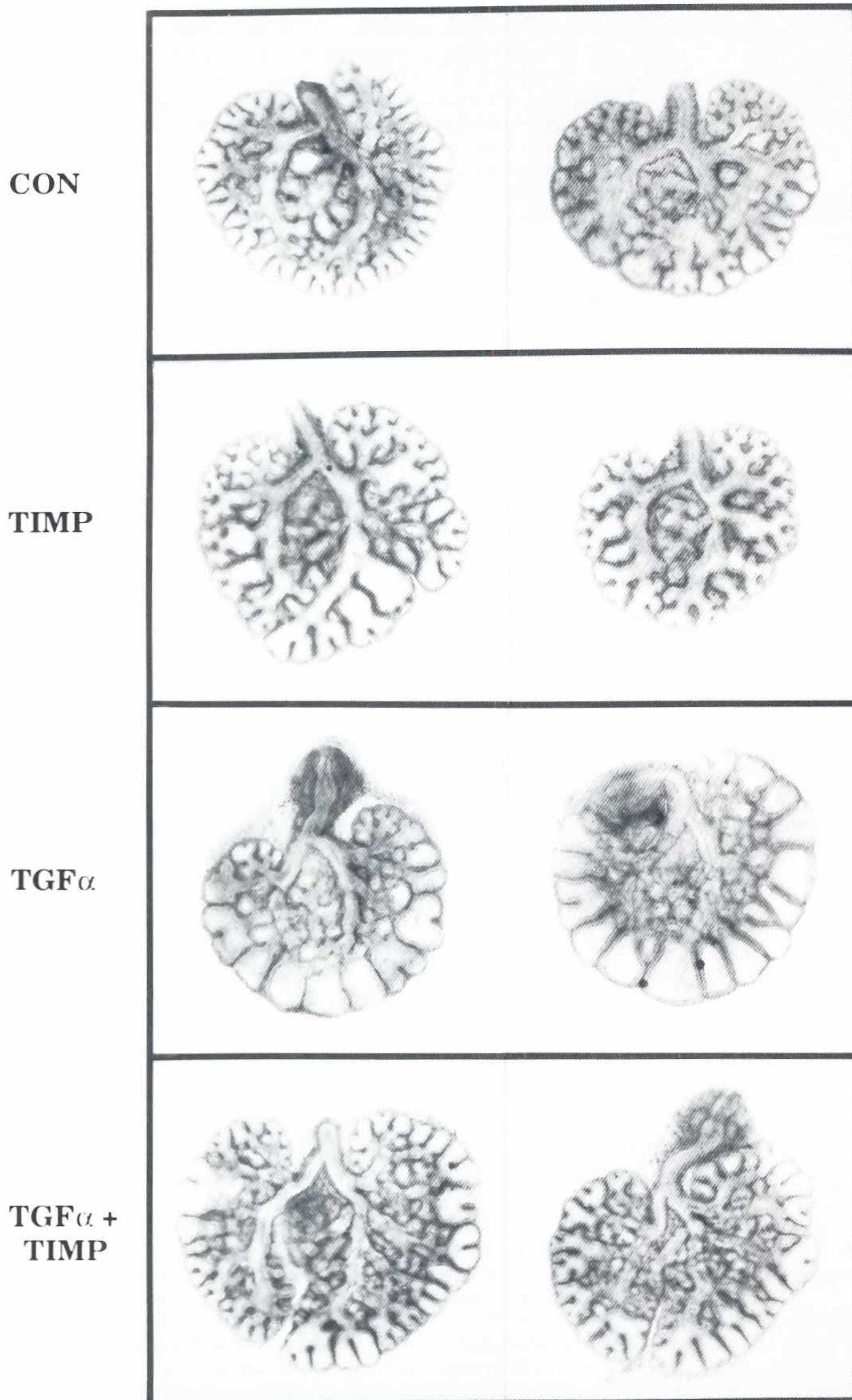

Fig. 5. Effects of TIMP upon control and TGFα-treated lung explants. *Lungs rudiments were maintained in culture for 3 days with daily addition of TIMP (50µg/ml) and TGFα (10 ng/ml) or no addition (CON) as indicated. Duplicate samples are shown and are representative of 3-4 lungs per group.*

TABLE 1

ANALYSIS OF THE AREA AND BRANCH DENSITY OF CONTROL LUNGS AND RUDIMENTS TREATED WITH TIMP, TGFα, OR BOTH TIMP AND TGFα

	Area (mm^2) mean±S.D.	Branches/mm^2 mean±S.D.
Control	3.30 ± .21	24.1± 2.3
TIMP	3.48 ± .68	23.9± 4.2
TGFα	3.70 ± .41	15.2± 2.6
TGFα+TIMP	4.05 ± .55	25.8± 3.8

Values for the area and branch density of lungs depicted in Fig. 5 were obtained as described in Materials and Methods. The total area of rudiments is the average of 3-4 individual rudiments. The value for the number of branches/mm^2 represents the mean of 3-7 independent measurements of 3-4 lungs.

enzyme is due to collagen degradation (see below). However, we cannot rule out the possibility that exogenous collagenase affects lung branching in an indirect manner, *e.g.*, by the release of growth factors from the ECM, rather than by a direct effect on matrix degradation. Several proteins, including the matrix protein laminin (Panayotou *et al.*, 1989), contain EGF-like repeats which may possess biological activities that could be unmasked by the action of proteases. Some growth factors, such as FGFs (Gospodarowicz, 1990) and TGFß (Yamaguchi *et al.*, 1990), are known to be matrix associated and may be released by the action of proteases. These growth factors may, in turn, have effects on the production of a variety of matrix metalloproteinases by the surrounding cells. For example, EGF has been shown to stimulate stromelysin expression (Kerr *et al.*, 1988), FGF to stimulate interstitial collagenase expression (Edwards *et al.*, 1987), and TGFß to stimulate type IV collagenase/gelatinase activity (Overall *et al.*, 1989) in some cell types. Since TIMP inhibits the activity of each of these metalloproteinases, our data suggest that the ultimate action of any one these metalloproteinases or a combination of them may contribute to the branching process. In these studies, however, we were unable to detect type I collagenase activity on substrate gels or by incubation of APMA-activated conditioned medium with [^{3}H]-type I collagen (data not shown). In addition, no stromelysin-like activity was detected on casein substrate gels (data not shown). We cannot, however, rule out a contribution by these MMPs since the site of synthesis or the limited sensitivity of the techniques employed may have precluded their detection in cultured lung conditioned medium. Thus, although the precise MMP and target substrate in our system have not yet been identified, these studies suggest that stringent control of metalloproteinase activity and extracellular matrix degradation are critical for normal lung branching and morphogenesis.

In this study, the end buds of TGFα-, EGF-, and collagenase-treated rudiments were broadened to similar degrees. However, growth factor-treated lungs were consistently larger than either control or collagenase-treated rudiments as determined by the overall area occupied by photographed specimens. This study does not differentiate whether this effect is due to an increase in cell proliferation or is secondary to the increase in size of the distal lumina. A growth factor-induced increase in proliferation would be consistent with the findings of Goldin and Opperman (1980), who demonstrated the EGF stimulation of DNA synthesis in embryonic chick lung trachea and bronchial epithelium. In the same study, EGF-containing agarose pellets were shown to induce supernumerary tracheal buds. It was therefore suggested that the characteristic branching pattern of the lung is achieved by localized increases in epithelial proliferation at presumptive bud sites. Such proliferation-driven branching has been proposed to guide the development of several branched organs (see Spooner and Thompson-Pletscher, 1986).

In the case of the lung and salivary gland, however, much evidence supports a model whereby branching is predominantly regulated not by differential proliferation but by cleft formation in distal epithelial buds. According to this model, matrix accumulates and restricts epithelium at forming clefts, whereas adjacent unrestricted epithelium continues to expand, yielding branches. In support of this theory, early autoradiographic studies failed to reveal any increase in the percentage of [^{3}H]-thymidine labeled nuclei in budding epithelium relative to adjacent non-budding epithelium (Wessells, 1970). Nakanishi *et al.* (1987) have reported continued cleft formation and branching in submandibular rudiments despite a substantial inhibition of DNA synthesis by X-ray irradiation. Furthermore, Spooner *et al.* (1989) report that tunicamycin treatment of embryonic salivary gland rudiments *in vitro* inhibits growth but not epithelial branching, yielding rudiments which are smaller but just as highly branched as controls. Other studies have shown that inhibitors of collagen or proteoglycan synthesis allow continued epithelial expansion but prevent normal branching morphogenesis (Spooner and Faubion, 1980; Spooner *et al.*, 1985). These findings, along with the results of this study which extend those of others (Grobstein and Cohen, 1965; Wessells and Cohen, 1968; Nakanishi *et al.*, 1986), suggest that, although a contribution of cellular proliferation cannot be entirely ruled out, it is the remodeling of the extracellular matrix that predominantly controls branching morphogenesis.

Gene products involved in the regulation of branching patterns are for the most part uncharacterized. Daniel and colleagues have proposed that TGFß1 is a natural regulator of mammary ductal growth, and that its effect is associated with the ability of this growth factor to stimulate synthesis of ECM components (Daniel *et al.*, 1989; Silberstein *et al.*, 1990). The data presented in this work support a model whereby metalloproteinase expression is involved in the development of characteristic epithelial trees (Nakanishi, *et al.*, 1986). This model implies that collagenolytic enzymes produced at regions of budding epithelium would participate in the rapid matrix degradation required for epithelial branch formation. Cleft regions, on the other hand, would be characterized by low proteolytic levels and/or an abundance of TIMP. Thus, it is most likely the net proteolytic balance of a specific region, determined by relative activities of MMPs and their specific inhibitors, as well as the amount and type of ECM present, that influences whether a given epithelial region will participate in branch or in cleft formation. Matrix-degrading enzymes could be produced by either branching epithelial cells themselves or the surrounding mesenchyme. Additional studies will be needed to identify the specific cell types producing metalloproteinases in response to growth factor stimulation. Most importantly, this study provides new evidence that developmentally expressed growth factors, such as EGF and TGFα, influence epithelial branching by modulating metalloproteinase

activities. In this manner, growth factors may help orchestrate the complex process of matrix remodeling, not only in branching tissues but during the development of multiple other organs as well.

Materials and Methods

Organ cultures

All embryos were obtained from ICR outbred females (Harlan Sprague-Dawley) mated with Swiss Webster males (Taconic Farms). Noon on the day of vaginal plug is 0.5 day *post coitum* (p.c.).

Lung organ cultures were carried out as previously described (Hirai, *et al.*, 1989) with the following modifications. Lung rudiments were dissected from 11.5 day p.c. fetuses. Lungs, either singly or in pairs, were cultured on nucleopore filters of 8 mm pore size and 13 mm diameter (Thomas Scientific) in 24-well plates. Culture medium was a 1:1 mixture of DMEM (high glucose, no pyruvate) and Ham's F12 (with 5 mg/L $CaCl_2$, JR Scientific), containing 1 µg/ml BSA, 10µg/ml human transferrin (GIBCO), and 50 µg/ml gentamicin. The addition of transferrin was found to be essential for the observed growth factor effects on lung morphogenesis. Dishes were maintained at 37°C in a humidified 95% air/5% CO_2 incubator. Growth factors were diluted in growth medium and initially added 2-4 hours after explanting, followed by once each day for the remainder of the experiment. Unless otherwise stated, no medium changes occurred during the culture period. EGF was from Amgen Biologicals; recombinant human TGFα was generously provided by Triton Biosciences, Alameda, California.

For photography, lungs were removed from the nucleopore filters and placed directly onto glass slides. Lungs were photographed under bright field of a Zeiss Axiophot microscope using either Kodak Panatonic-X or T-Max 100 film. Lung branching was quantitated by counting end buds present in a defined area of the lung rudiments (usually 1/3 to 1/2 of the lung, the area of which was measured morphometrically as described below). Occasionally, there were instances where a newly forming cleft had just begun to separate one bud into two buds. If the length of the new cleft (from its central to peripheral edge) exceeded half the greatest width of each newly forming bud, then the segment was considered to have branched and was counted as two buds.

Both total and defined lung areas were determined by using a Microcom image analysis system (Southern Micro Instruments) integrated with an IBM AT computer. Lung areas were determined from photographs and then corrected for photographic magnification so that the final value represents the size of the original lung when viewed in two dimensions. The trachea was not included in area measurements.

For histological examination, lung rudiments were removed from nucleopore filters, fixed in 95% formalin, and embedded in paraffin wax. Sections (5 µm) were stained with Mayer's hematoxylin and eosin, and analyzed on a Zeiss Axiophot microscope.

Collagenase and TIMP

Human recombinant TIMP was supplied by Synergen, Inc., Boulder, CO. Its cloning and use have been described (Carmichael *et al.*, 1986, 1989). TIMP was added to lung cultures daily where indicated.

Human fibroblast collagenase was obtained in pure form from serum-free conditioned medium harvested from phorbol 12-myristate 13-acetate (PMA) stimulated SV40 transformed fibroblasts selected for enhanced production of collagenase (cells kindly provided by Dr. Zena Werb). The medium was clarified by centrifugation and filtration, made 10 mM Tris-HCL, pH 7.5 and 5 mM $CaCl_2$, and applied to a 2.6 x 5 cm column of zinc chelate Sepharose (Pharmacia) equilibrated in the same buffer. Bound protein was eluted in a stepwise fashion with 0.25 M Imidazole, pH 7.5 with 0.01 M $CaCl_2$ and dialyzed against large volumes of 0.01 M Tris-HCl, pH 7.5 overnight at 4°C. The eluent pool was clarified by centrifugation and filtration, and applied to a MonoS column (Pharmacia) equilibrated in 0.01 M MOPS, pH 7.2 with 0.05% Brij-35 (MOPS-Brij-35). Bound proteins were eluted by a linear gradient of NaCl in MOPS-Brij-35 at approximately 0.3 M NaCl. Purity was established by SDS-PAGE and Western blot and found to be equivalent to that produced by earlier chromatographic methods (Stricklin *et al.*, 1974, 1978). Prior to use, collagenase was activated by the addition of trypsin at a 1:100 weight ratio of trypsin:collagenase and incubation at 37°C for 1 hour. Trypsin inhibitor was then added to a final concentration of 0.5 µg/µl. Collagenase was diluted in the growth medium and added to lungs as a single dose at 2-4 hours after explanting. Bacterial collagenases used in some experiments were Collagenase CLS-3 from Worthington Biochemical Corporation and Collagenase Form III from Advance Biofactures Corporation.

Gelatin-substrate enzymography

Lung rudiments were cultured for 3 days with or without the daily addition of TGFα or EGF. Each day, the conditioned medium was collected and concentrated approximately 20-fold using Centricon-10 microconcentrators (Amicon). Samples were loaded onto a 10% SDS-polyacrylamide gel containing 0.75 mg/ml bovine skin gelatin (Sigma, G9382). Following electrophoresis, the gel was washed twice for 15 minutes in 2.5% Triton X-100 and incubated overnight at 37°C in substrate buffer containing 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0. For inhibition of metalloproteinase activity, 10 mM EDTA was included in the substrate buffer. The gel was then stained with 0.5% Coomassie Blue R250 in 50% methanol/10% acetic acid for 45 minutes, followed by overnight destaining in methanol/acetic acid.

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