INFORMATION TO USERS

This reproduction was made from a copy of a manuscript sent to us for publication and microfilming. While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. Pages in any manuscript may have indistinct print. In all cases the best available copy has been filmed.

The following explanation of techniques is provided to help clarify notations which may appear on this reproduction.

1. Manuscripts may not always be complete. When it is not possible to obtain missing pages, a note appears to indicate this.

2. When copyrighted materials are removed from the manuscript, a note appears to indicate this.

3. Oversize materials (maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or in black and white paper format.*

4. Most photographs reproduce acceptably on positive microfilm or microfiche but lack clarity on xerographic copies made from the microfilm. For an additional charge, all photographs are available in black and white standard 35mm slide format.*

*For more information about black and white slides or enlarged paper reproductions, please contact the Dissertations Customer Services Department.
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages
2. Colored illustrations, paper or print
3. Photographs with dark background ✓
4. Illustrations are poor copy
5. Pages with black marks, not original copy ✓
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages
15. Dissertation contains pages with print at a slant, filmed as received ✓
16. Other

University Microfilms International
LUNG-DERIVED GROWTH FACTORS:
POSSIBLE PARACRINE EFFECTORS OF FETAL LUNG DEVELOPMENT

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN BIOMEDICAL SCIENCES (PHYSIOLOGY)

DECEMBER 1985

By
Ana Maria Montes

Dissertation Committee:
Walter K. Morishige, Chairman
John R. Claybaugh
Martin D. Rayner
G. Causey Whittow
Samuel R. Haley
ACKNOWLEDGEMENTS

For his invaluable guidance as a scientist, patience as a mentor, and support as a friend, I would like to gratefully acknowledge the help of Dr. Walter Morishige.
ABSTRACT

Morphogenesis involves the specific arrangement of embryonic cell populations which ultimately results in organs with unique structures. Substantial evidence suggests that cell and tissue interactions are the primary regulatory mechanisms for the developmental process. A potential role for paracrine secretions in lung organogenesis has been hypothesized (Alescio & Piperno, 1957). These studies present direct support for the paracrine model by demonstrating the presence of locally produced mitogenic/maturational factors in fetal rat lung tissue.

Conditioned serum free medium (CSFM) from nineteen-day fetal rat lung cultures was shown to contain several bioactive peptides as detected by $^3$H-Thymidine incorporation into chick embryo and rat lung fibroblasts, as well as $^{14}$C-choline incorporation into surfactant in mixed cell cultures. Using ion-exchange chromatography and Sephadex gel filtration, a partially purified mitogen, 11-III, was obtained. Although 11-III cross-reacts in the somatomedin-C (Sm-C) radioimmunoassay, disc polyacrylamide gel electrophoresis demonstrates that this component has an electrophoretic mobility [$R_f$ value] of 0.59 (+/-0.02) and does not co-migrate with Sm-C in this system ($R_f = 0.53$) and, thus, is probably not authentic Sm-C. The
partially purified II-III stimulates mitosis in chick embryo fibroblasts and post-natal rat lung fibroblasts. Multiplication in fetal rat lung fibroblast cultures is stimulated only when these are pre-incubated with a competence factor or unprocessed CSFM. This suggests the existence of an endogenously produced competence factor important in the regulation of fetal lung growth. Preparation II-III does not possess surfactant stimulating activity as assessed by $^3$H-choline incorporation into lipids in predominantly type-II cell cultures. However, a peak of surfactant stimulating activity was found to elute off Sephadex G-75 at a $K_aV = 0.23$ (MW approx. 13 Kd) and was distinct from the mitogenic II-III peak ($K_aV = 0.50$).

These data demonstrate the presence of a maturational/ mitogenic factor, influencing type-II mixed cell cultures. In addition, II-III had been shown to play an autocrine role stimulating the proliferation of fetal lung fibroblasts. Finally, these data suggest the existence of a local produced competence factor.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>GROWTH</td>
<td>1</td>
</tr>
<tr>
<td>Role of Systemic Hormones in Growth</td>
<td>2</td>
</tr>
<tr>
<td>Potential Role of Placental Hormones</td>
<td>5</td>
</tr>
<tr>
<td>Role of Growth Factors</td>
<td>6</td>
</tr>
<tr>
<td>Biochemical Characterization of Somatomedins</td>
<td>7</td>
</tr>
<tr>
<td>Evidence for Somatomedin Regulated Growth</td>
<td>10</td>
</tr>
<tr>
<td>Regulation of the Cell Cycle</td>
<td>12</td>
</tr>
<tr>
<td>Role of Hormones in Lung Development</td>
<td>14</td>
</tr>
<tr>
<td>Statement of the Problem</td>
<td>20</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>23</td>
</tr>
<tr>
<td>Isolation of Lung Derived Peptides</td>
<td>23</td>
</tr>
<tr>
<td>Animals</td>
<td>23</td>
</tr>
<tr>
<td>Organ Explant Cultures</td>
<td>23</td>
</tr>
<tr>
<td>Peptide Isolation</td>
<td>25</td>
</tr>
<tr>
<td>Ion Exchange Chromatography</td>
<td>25</td>
</tr>
<tr>
<td>Sephadex Gel Filtration</td>
<td>26</td>
</tr>
<tr>
<td>Polycrylamide Gel Electrophoresis</td>
<td>26</td>
</tr>
<tr>
<td>Protein Determination</td>
<td>27</td>
</tr>
<tr>
<td>Radioimmunoassay</td>
<td>27</td>
</tr>
<tr>
<td>Biological Characterization</td>
<td>28</td>
</tr>
<tr>
<td>Chick Embryo Fibroblast Assay</td>
<td>28</td>
</tr>
<tr>
<td>Rat Lung Fibroblast Assay</td>
<td>29</td>
</tr>
<tr>
<td>Choline Bioassay</td>
<td>31</td>
</tr>
<tr>
<td>Statistics</td>
<td>32</td>
</tr>
<tr>
<td>RESULTS</td>
<td>33</td>
</tr>
<tr>
<td>Partial Isolation of Lung Derived Mitogen</td>
<td>33</td>
</tr>
<tr>
<td>Ion Exchange Chromatography</td>
<td>33</td>
</tr>
<tr>
<td>Gel Filtration</td>
<td>39</td>
</tr>
<tr>
<td>Dose Response Studies</td>
<td>42</td>
</tr>
<tr>
<td>Analytical Disc Acrylamide Gel Electrophoresis (PAGE)</td>
<td>47</td>
</tr>
<tr>
<td>Acid PAGE of $^{125}$I-Somatomedin-C</td>
<td>50</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Isolation of Mitogen from Fetal Lung Cell Conditioned Serum-Free Medium</td>
<td>38</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Isolation Scheme</td>
</tr>
<tr>
<td>2</td>
<td>Cation Exchange Chromatography of Conditioned Serum-Free Medium</td>
</tr>
<tr>
<td>3</td>
<td>Sephadex G-75 Chromatography of the pH 11 Preparation</td>
</tr>
<tr>
<td>4</td>
<td>Measurement of Immunoreactivity of Lung-Derived Factors by the Somatomedin-C Radioimmunoassay</td>
</tr>
<tr>
<td>5</td>
<td>Measurement of Bioactivity of Lung-Derived Factors in the Chick Embryo Fibroblast Assay</td>
</tr>
<tr>
<td>6</td>
<td>Electrophoretic Profiles of II-II and II-III</td>
</tr>
<tr>
<td>7</td>
<td>Bioassay and Radioimmunoassay of II-III Electrophoretic Profile</td>
</tr>
<tr>
<td>8</td>
<td>Electrophoretic Profile of I₂⁵-I-Somatomedin-C</td>
</tr>
<tr>
<td>9</td>
<td>Effect of II-III on (^3)H-Thymidine Incorporation into Postnatal Rat Lung Fibroblasts</td>
</tr>
<tr>
<td>10</td>
<td>Effect of II-III on (^3)H-Thymidine Incorporation into Fetal Lung Fibroblasts</td>
</tr>
<tr>
<td>11</td>
<td>Effect of II-III on (^3)H-Thymidine Incorporation into Postnatal Day 4 Rat Lung Fibroblasts: Evidence for Endogenously Produced Growth Factor</td>
</tr>
<tr>
<td>12</td>
<td>Effect of II-III on (^3)H-Thymidine Incorporation into Fetal Day 19 Rat Lung Fibroblasts: Evidence for Endogenously Produced Competence Activity</td>
</tr>
<tr>
<td>13</td>
<td>Fetal Lung Fibroblasts Require Exposure to Competence Activity for Responsiveness to II-III: Evidence that II-III is a Pure Progression Factor</td>
</tr>
</tbody>
</table>
14 Mitogenic Response of Postnatal Day 4 Rat Lung Fibroblasts to II-III in the Presence of Epidermal Growth Factor (EGF) 69

15 Photomicrographs of Rat Lung Fibroblast Cultures 72

16 Photomicrographs of Mixed Rat Lung Cell Cultures 74

17 Photomicrographs of Mixed Cell Cultures 76

18 Effect of Cortisol on Phospholipid Synthesis in Primary Mixed Lung Cell Cultures 79

19 Effect of II-III on Phospholipid Synthesis in Primary Mixed Lung Cell Cultures 81

20 Phospholipid Synthesis in the A549 Cell Line: Effects of Cortisol and II-III after 24 hours of Exposure 83

21 Phospholipid Synthesis in the A549 Cell Line: Effects of Cortisol and II-III after 48 hours of Exposure 87

22 Sephadex G-75 Chromatography of Conditioned Serum-Free Medium: Evidence for a Surfactant Stimulating Factor Distinct from II-III 89
GROWTH

Growth is the most evident and striking component in the development of a multicellular organism as it passes from a single fertilized ovum into an adult. Strictly defined, growth denotes a change in size due to an increase in the number or size of cells. Growth as such may be analyzed in terms of cell multiplication, cell enlargement, cell replacement, and other features such as accumulation of extracellular substance. It is interesting, however, that no sharp distinction can be made between cellular processes that maintain an organism's size and those that increase it. Growth processes which increase tissue mass or an organism's size can be seen as a shift away from equilibrium.

Of all the processes involved in growth perhaps the single most important mechanism is that of cell proliferation. Early prenatal growth in the rat proceeds entirely by cell division. Increases in whole animal DNA closely match increases in body weight and protein accretion during the fetal period (Enesco and Leblond, 1962; Winick and Noble, 1965). In individual organs, beginning at day 10 of gestation and proceeding for variable periods, growth can be entirely attributed to mitosis. This period of rapid mitotic growth is followed
by a period when cell division and cell enlargement increase simultaneously. Finally, the last stage of organ growth is due to cell enlargement in the form of protein accumulation and extracellular protein deposition. Specific rates of mitosis are related to specific stages in developmental growth. Skin provides a relatively simple model which exemplifies how cell division is modulated during development at the organ level.

In its earliest stages a single layer of cells constitutes the skin. As the embryo increases in size the surface layer must increase and cells divide synchronously resulting in an exponential rate of organ growth. As the organism matures, layers of quiescent cells develop superficial to the proliferating population. As the basal germinal layer differentiates, the numbers of nascent cells relative to the total number of skin cells decreases. Cell proliferation becomes asynchronous and at this stage organ growth becomes a linear process.

Role of Systemic Hormones in Growth

The control of growth involves the harmonious coordination of a myriad of factors: genetic, environmental, and hormonal, to name a few. This paper will be limited to discussing the hormonal regulation of growth.

Systemic hormones have been shown to play a
significant role in postnatal growth. Growth hormone and thyroid hormones play a major growth promoting role since hypophysectomy and thyroidectomy both dramatically decrease growth rates in neonatal rats (Glasscock and Nicoll, 1981). Growth hormone (GH) or somatotropin promotes linear growth and its deficiency or excess will result in growth retardation and acromegaly respectively. Insight into somatotropin's mechanism of action began when Salmon and Daughaday (1957) found that while GH stimulated in vivo incorporation of sulfate into chondrocytes, it was inactive in vitro. They presented evidence for a factor in serum under the control of GH that stimulated in vitro sulfate incorporation into cartilage. This substance they called "sulfation factor". Further research revealed that this factor stimulated the synthesis of DNA, RNA, protein and hydroxyproline in chondrocytes. Since this factor mediated the actions of somatotropin in the adult it was renamed somatomedin. Several somatomedins have since been identified.

There is evidence, however, to indicate that perinatal and neonatal animals are far less responsive to thyroid and growth hormone than juvenile and adult animals (Glasscock and Nicoll, 1981). This suggests that the regulatory role of the 'classical' hormones may change throughout development, becoming progressively more prominent with age. In addition, it suggests that other
"fetal hormones" may be playing the major regulatory role during intrauterine development.

In the fetus the role of systemic hormones has been difficult to establish. In the fetal rat, rapid growth of the adrenal glands occurs at day 16 of gestation and plasma concentrations of corticosterone (the major glucocorticoid in this species) peaks at day 18 or 19 (Kamoun, 1970; Nathanielz, 1976). In rabbit and sheep glucocorticoid levels increase until term and decline postnatally. Glucocorticoids have been implicated in various maturational processes in fetal development such as precocious surfactant production in the lung and development of glucogenic enzymes in the liver, and is not associated with increased fetal growth. In addition, although cortisol stimulates a variety of maturational events it has inhibitory effects on mitosis (Jimenez de Asua, et al., 1977) and appears to inhibit growth in primates and man (Reinisch, et al., 1978).

Similarly, evidence exists that thyroid hormones play a maturational rather than a growth promoting role in the fetus. In primates and man, hypothyroidism is similarly associated with normal birth size (Kerr, et al., 1975). It is established, however, that thyroid hormones promote differentiation in bone, the nervous system, the skin, and the lung.
In the fetus, GH appears late in gestation and levels remain elevated until parturition in most mammals. In the rat, GH is first detected in the pituitary and plasma at 19 days of gestation (parturition = 22 days). On gestation day 21.5 the plasma concentration has more than doubled and peaks immediately after birth. By postnatal day 10 the levels have decreased to adult values (Rieutort, 1974). The significance of these high fetal and perinatal levels is unknown; similar patterns, however, have been observed in sheep and man. Nevertheless, it is believed that GH is of minimal importance in fetal growth since anencephaly, resulting in GH deficiency, is associated with normal or near normal size at birth in man, rat, mouse, sheep, and rabbit (Chen, 1954; Eguchi, 1961; Reid, 1978).

**Potential Role of Placental Hormones**

The placenta synthesizes several peptides that are structurally and biologically homologous with the hypothalamic-hypophyseal hormones. Included in this list is placental lactogen (PL) which is a product of the fetal syncytiotrophoblast. It has been shown to have an 85% homology with human GH. In vivo studies have demonstrated that ovine PL is equipotent with ovine and bovine GH in inducing Somatomedin-C in the serum of hypophysectomized rats (Hurley, et al., 1977). In humans, immunoreactive
Somatomedin-C (Sm-C) rises progressively during pregnancy and correlates with maternal PL levels (Furlanetto, et al., 1978). Low maternal human PL concentrations are associated with decreased fetal growth (Cramer, et al., 1971). This growing body of literature has led to the speculation that PL rather than GH is the prime regulator of somatomedin in fetal development (Underwood, et al., 1979). In addition, Daughaday and Kapadia (1978) demonstrated that bioactive somatomedin levels did not decline in hypophysectomized pregnant rats although the levels fell immediately after delivery. This supports speculation that PL regulates maternal somatomedin levels during pregnancy (Underwood and D'Ercole, 1984). Recently, Adams et al., (1983a) have reported that ovine PL stimulates in vitro synthesis of rat insulin-like growth factor II (IGF-II) by tertiary fetal rat fibroblasts while GH was inactive. Interestingly, when adult rat fibroblasts were used, Sm-C (not IGF-II) synthesis was stimulated by both ovine PL and GH (Adams, et al., 1983a). These data would indicate not only that somatomedin production is under PL regulation, but that there is, in addition, a "fetal" somatomedin which predominates during in utero growth.

**Role of Growth Factors**

In recent years a large amount of evidence has
accumulated to indicate that some growth factors and the somatomedins play an important role in mammalian growth. Somatomedins have been detected in the fetal circulation of many mammalian species and it is speculated that these hormones influence body growth in utero. Circulating somatomedin levels at term correlate positively with gestational age and fetal body weight (Gluckman and Brinsmead, 1976). In the fetus, however, GH appears not to be playing a role in regulating the somatomedin levels as GH-deficient children have normal birth weight. In the rat or rabbit decapitation in utero results in no reduction in plasma somatomedin activity. Injection of insulin, however, did cause an increase in somatomedin activity (Hill and Milner, 1983). Somatomedins in cord blood represent somatomedin of fetal origin as it has been shown that somatomedin-C (Sm-C) does not cross the placenta (Underwood, et al., 1979; D'Ercole, et al., 1980). Sm-C appears to be synthesized de novo by the mouse from as early as day 11 of gestation (D'Ercole and Underwood, 1980).

**Biochemical Characterization of the Somatomedins**

Subsequent research in this area revealed that human serum contained several somatomedins. Somatomedin C (Sm-C) is a basic polypeptide of 7,500 daltons. Somatomedin A (Sm-A) is a neutral polypeptide which has not been well
defined biochemically.

Independent studies on the nature of insulin-like activity in human serum revealed that addition of excess insulin antibody to serum suppressed only 10% of the insulin-like activity (Froesch, et al., 1963). The nonsuppressible insulin-like activity (NSILA) was found to possess somatomedin-like biological properties. Two peptides, insulin-like growth factor I (IGF-I) and IGF-II, were subsequently isolated from human serum and shown to be responsible for the NSILA. All the aforementioned peptides are ancestrally related to proinsulin (Van Wyk and Underwood, 1978) and, thus, are often referred to as insulin-like growth factors. Somatomedin-C and IGF-I, both basic peptides, presently have provisionally identical amino-acid sequences (Svoboda, et al., 1980). IGF-II, however, has only 70% homology with Sm-C. In addition, Sm-C is more growth hormone dependent and more active in stimulating the incorporation of sulfate into cartilage than is IGF-II (Zapf, et al., 1978). Thus, presently it is known that three distinct somatomedins exist in human serum: Sm-C (IGF-I), IGF-II and, Sm-A.

Rat Somatomedins

In the rat, there appears to be more than one form of somatomedin as well. Multiplication stimulating activity
(MSA), originally isolated by Peirson and Temin (1971), has been shown to be a family of closely related peptides synthesized by a buffalo rat liver cell line (BRL-3A) in culture. Buffalo rat liver MSA (BRL-MSA) closely resembles the somatomedins found in human serum both in biological and biochemical properties. They share with the somatomedins their reactivity with somatomedin-binding proteins in human and rat serum and their interaction with specific cell-surface receptors (Rechler, et al., 1980). At least three separate MSA peptides exist. MSA-I has a molecular weight of 16,300 and an electrophoretic mobility ($R_f$) of 0.36 in acid (pH=2.7) urea (9M) disc acrylamide (12.5%) electrophoresis system. MSA-II has a molecular weight of 8,700 and an $R_f$ value of 0.41-0.49. MSA-III, 7,100 daltons, has an $R_f$ value of 0.59-0.62. This latter peptide has been shown to have a 93% homology with human IGF-II (Marquardt and Todaro, 1981).

In summary, to qualify as a somatomedin a peptide must fulfill the following biological criteria: 1) enhance sulfate incorporation into cartilage 2) exert insulin-like effects on extraskeletal tissues, 3) stimulate the multiplication of a variety of cultured cells in serum free medium and, 4) be growth hormone dependent. The family of somatomedins presently includes: somatomedin- A, Sm-C/IGF-I, insulin-like growth factor I (IGF-I), insulin-
like growth factor II (IGF-II) and the MSA family of growth peptides.

**Evidence for Somatomedin Regulated Growth**

Although somatomedins were first described as skeletal growth factors, it is now known that specific receptors for Sm-C (D'Ercole, *et al*., 1976a) and MSA (Daughaday, *et al*., 1982; Owen, *et al*., 1980) are ubiquitously distributed on mammalian tissues. This agrees with the fact that the somatomedins stimulate thymidine incorporation into DNA in a variety of cell types (Van Wyk, *et al*., 1981). In addition, it has been shown that the quantity and affinity of Sm-C receptors are greater in the fetus than in the adult (D'Ercole, *et al*., 1976b). It is significant that in some tissues, such as the fetal placenta and the lung, the apparent number and/or affinity of these receptors was significantly higher than in adult tissues or early gestation tissues.

In addition to the seemingly ubiquitous distribution of somatomedin receptors, there is also evidence that somatomedins are synthesised in multiple fetal tissues other than the liver (D'Ercole and Underwood, 1980). Immunoreactive somatomedin-C can be detected in the conditioned serum free medium of human skin fibroblasts (Clemmons, *et al*., 1981), the WI-38 cell line, a cloned line of embryonic human lung fibroblasts (Atkinson, *et*
al., 1980) and, as previously mentioned, tertiary cultures of adult rat fibroblasts (Adams, et al., 1983a). In these systems GH stimulates immunoreactive somatomedin production in a dose dependent manner.

Recent studies by Adams, et al., (1983a) have demonstrated two classes of specific growth peptide receptors in tertiary fetal rat fibroblasts. One of these identifies MSA and IGF-II; the other identifies IGF-I. Additional studies (Adams, et al., 1983b) demonstrated that third passage fibroblasts from 16 day old rat fetuses synthesized a small molecular weight polypeptide that was indistinguishable from BRL-MSA polypeptides as determined biochemically (HPLC and disc electrophoresis), by behavior in RIA, bioassay, receptor binding assay and competitive protein binding assay. These findings are particularly significant in light of the fact that IGF-II/MSA levels are 20 to 100 fold higher in fetal rat serum than in maternal serum and decline within days after birth (Moses, et al., 1977). IGF-I levels, on the other hand, are lower in fetal and neonatal serum than in maternal serum and increase with age (Daughaday, et al., 1982). Shortly thereafter, Adams, et al., (1983a) using tertiary fetal fibroblasts, presented evidence for a developmental switch in growth factor production in vitro which correlates with the in vivo pattern. These data support the idea that the somatomedins are playing a vital role in fetal growth and
that the regulatory mechanisms of fetal growth are distinct from those involved in postnatal growth.

**Regulation of the Cell Cycle**

Since the discovery of somatomedins it had been observed that somatomedins by themselves could not fully substitute for serum in promoting cell growth. Although somatomedins stimulate incorporation of thymidine into DNA in embryonic chick fibroblast *in vitro*, the magnitude of the response is lower than when fetal bovine serum is added to the medium (Smith and Temin, 1974; Moses, *et al.*, 1978). Similar studies using human fibroblasts, however, demonstrated that mammalian cells *in vitro* do not respond to pure preparations of somatomedins (Moses, *et al.*, 1978; Antoniades, *et al.*, 1975). Studies by Ross, *et al.*, (1974) demonstrated that monkey blood serum promoted the proliferation of monkey arterial smooth muscle cells in culture while platelet-poor plasma was much less effective. Addition of platelets to platelet-poor plasma increased the activity to match blood serum activity, demonstrating that much of the growth promoting activity of dialysed serum is derived from platelets. This platelet derived growth factor (PDGF) was later shown to be a 13,000 dalton basic polypeptide (Antoniades, *et al.*, 1975; Antoniades, *et al.*, 1979). These studies explained the discrepancy of response to growth factors in avian
versus mammalian cells. Further studies revealed that the stimulatory action of serum was shown to be due to the sequential action of PDGF followed by the action of growth factors in the medium (Pledger, et al., 1977). This work, which was done using BALB/c 3T3 cell line, a murine fibroblast of embryonic origin, suggested that certain factors were necessary in moving cells through the prereplicative phase of the cell cycle. These factors were termed "competence factors" since progression through G₁ in response to growth factors could occur only if resting cells were first made "competent" (Van Wyk, et al., 1981). When PDGF is removed from the plasma the fibroblasts will not progress to the synthetic phase (S) of the cell cycle. Stiles, et al., (1979) demonstrated that competent BALB/c 3T3 cells will not progress to the S phase in 3% hypopituitary plasma which is known to contain PDGF. Thus, there appears to be dual control of cell growth by "competence factors", such as PDGF, and "progression factors", such as the somatomedins.

A distinct competence factor, fibroblast growth factor (FGF), was isolated and purified from bovine pituitary glands by Gospodarowicz (1975). In addition, studies have shown that while FGF is mitogenic for mesodermal cells in vitro the factor is not active when used with endodermal or ectodermal cells (Gospodarowicz
and Moran, 1976b; Scher, et al., 1980). Although FGF can stimulate DNA synthesis in sparse resting 3T3 cells, it cannot, by itself, replace the serum requirement of these cells (Gospodarowicz, et al., 1976b). It has also been shown that cells require only transient exposure to competence factors for DNA synthesis while progression factors are required continuously (Pledger, et al., 1977). This is true for both PDGF and FGF.

ROLE OF HORMONES IN LUNG DEVELOPMENT

Late fetal life is characterized by various developmental processes which ultimately prepare the fetus for extrauterine survival. Development of the neonate's respiratory system necessarily precludes adaptation to the extrauterine environment; thus, much attention has focused on lung development in an effort to understand the etiology and delineate preventive clinical treatment of hyaline membrane disease, or infant respiratory distress syndrome (RDS), as it is more commonly referred to today. This condition, characterized by respiratory insufficiency, is a disease confined to premature infants or predominantly those weighing less than 2500gm at birth (Singer, 1984). It is estimated that 40,000 infants per year develop RDS in the US alone (Vidyasagar, 1984).

The pathological characteristics of RDS were first
described by Hochheim in 1903 and more detailed clinical descriptions followed. The pathogenesis of RDS, however, remained obscure although atelectasis was recognised as the feature of major physiological significance in the disease. Insight into the pathogenesis of the disease came in the 1950's with the work of Cook, et al., (1957) who demonstrated that infants with neonatal respiratory distress have significantly lower pulmonary compliance, and the later studies by Avery and Mead (1959), who demonstrated that the surface tension of lung extracts of RDS infants was higher than expected. It subsequently became clear that RDS was a developmental disease.

As early as 1968 it was hypothesized that the endodermally derived fetal lung and gut both required glucocorticoids for proper development (Buckingham, et al., 1968). This was subsequently confirmed by Liggins (1969) who noted that preterm lambs treated with glucocorticoids were unexpectedly viable. The following year de Lemos, et al., (1970) clearly demonstrated that precocious fetal lung maturation occurred in glucocorticoid treated sheep. Thus, by the early 1970's the importance of pulmonary endocrinology in understanding and clinically treating RDS was recognized and efforts to elucidate the hormonal regulation of lung organogenesis had begun.
Since Liggins' (1969) initial observation that precocious lung maturation occurs as a result of antenatal glucocorticoid treatment a great number of studies have been done to determine the mechanism whereby these steroids exert their action. Glucocorticoids were also found to stimulate surfactant production in vitro in fetal lung organ cultures and in mixed cell cultures (Gross, et al., 1980, Gross, 1983). Cytoplasmic receptors to glucocorticoids have been identified in two populations of lung cells: pulmonary fibroblasts and alveolar type-II cells (Ballard, 1980). The morphological effects of antenatal glucocorticoid administration have been well documented and include flattening of epithelial cells, narrowing of septa, increased number of alveolae, and appearance of lamellar bodies (Hitchcock, 1980). Physiologically, there is greater distensibility, greater deflation stability, and earlier appearance of surface active material. Biochemically, the major effect of glucocorticoids on type-II cells is through increasing the concentration and degree of saturation of phosphatidylcholine (Hitchcock, 1980). In addition, glucocorticoid exposure results in increased enzymatic activity in surfactant synthesizing pathways. It is interesting to note that glucocorticoid therapy also results in decreased lung growth in rabbits (Carson, et al., 1973).
Receptors for thyroxine have been demonstrated in rat and Ballard, 1982). In addition, thyroxine has been shown to stimulate fetal lung maturation and acts synergistically with glucocorticoids to augment surfactant production (Gross, 1983). These hormones influence the rate of maturation although, it seems, not the pattern of differentiation. (Ballard, 1983). Maturation, as monitored by disaturated phosphatidyl choline accumulation, in organotypic fetal rat lung cultures in the absence of hormones has been reported to correlate well with in vivo biochemical changes and is not dependent on time in culture, suggesting that systemic hormones modulate but do not initiate differentiation (Gross, 1983).

In addition to the aforementioned hormones it was shown that peptide factors were agents involved in accelerating lung maturation. Injection of epidermal growth factor (EGF) into rabbit fetuses promotes both epithelial cell growth and cytodifferentiation in fetal lung (Catterton, et al., 1979). This factor, originally isolated from mouse salivary glands (Cohen, 1963), has been found to be mitogenic for a variety of epithelial tissues eliciting a classic pleiotropic effect (i.e., increased ion and precursor uptake, enhanced glycolysis, RNA, DNA, and protein synthesis). Sundell, et al., (1980)
demonstrated that infusions of EGF induced respiratory competence in premature lambs as early as day 128 of gestation. This demonstrated that the lung was responsive to growth factors and implicated a role for endogenous peptide factors.

As early as 1949 studies with lung rudiments began to indicate that the fetal lung was a self-developing entity (Waddell, 1949). In vitro organ explant studies later supported this idea (Chen, 1954). Culture studies have favored the idea that many of the morphological characteristics, particularly the branching epithelial tree, can develop normally in the absence of systemic influences (Rudnick, 1933; Sorokin, 1961; Alescio and diMichelle, 1968), although after several days in culture growth falls behind age matched controls as determined by epithelial and mesenchymal mitotic indices (Sorokin, 1961).

Dameron (1961) demonstrated that the epithelium of the fetal lung isolated from the surrounding mesenchyme was incapable of morphogenesis. When the epithelium is recombined with pulmonary mesenchyme development resumes. The inductive influence of mesenchyme on the growth of lung epithelium was recognized by Alescio and associates. Working with cultured lung rudiments of the mouse they found that if the epithelium/mesenchyme ratio was altered in favor of mesenchyme, epithelial growth was
significantly increased (Alescio and Piperno, 1967). This agreed with studies in other epithelio-mesenchymal organs as had been demonstrated by Grobstein (1953) in the fetal mouse salivary gland. It was speculated that a growth factor was responsible for the inductive lung influence and that such a factor could regulate mitosis and/or cytodifferentiation.

Smith reported in 1979 that glucocorticoids stimulated the production of a peptide factor by cultured fetal lung tissue. This peptide, fibroblast-pneumocyte factor, (FPF) having an approximate molecular weight of 6,000 daltons, was reported to stimulate surfactant production, as measured by the incorporation of radiolabeled choline into disaturated phosphatidylcholine. The existence of a fibroblast produced factor would explain why in vitro studies showed that glucocorticoids could stimulate surfactant production in fetal lung organ cultures and in mixed cell cultures (Gross, et al., 1980) while this stimulatory effect was lost when cloned type-II epithelial cells are used (Smith, 1979). In addition, there was some evidence to suggest that FPF stimulates surfactant production in pure type-II cell cultures and that FPF production varies with the gestational age of the fibroblasts cultured becoming maximal at day 20 of gestation. (Smith, 1981). It is significant that
morphologically distinct type-II cells are first seen at day 20 of gestation in the rat in vitro. These studies have led to speculation that a local peptide factor may mediate the maturational actions of glucocorticoids.

STATEMENT OF THE PROBLEM

Morphogenesis is the process whereby a tissue primordium arises and develops into a mature organ exhibiting a characteristic arrangement of cell populations each having specific functions. In epithelial-mesenchymal organs, such as the lung, final organ form is dependent on the interaction of the epithelial and mesenchymal components. It has been noted that evidence exists suggesting that lung mesenchyme is producing a factor or factors exerting both mitogenic and maturational effects. The question, then, regards the identity of the factor(s) produced by the fetal lung. Alescio and diMicelle (1968), in addressing this issue, have postulated the existence of a "mesenchymal factor" the mitogenic actions of which were directed at the epithelium. Smith (1979, 1981), on the other hand, has presented evidence for a non-mitogenic fibroblast derived factor which promotes the differentiated function of the epithelium. In neither study were the peptides purified and characterized. It would be important to isolate and characterize these factor(s) in order to determine whether
a single factor was responsible for both growth promoting and maturational effects. Moreover the mesodermally derived peptides of the above-mentioned studies were not examined for any effect on lung mesoderm. In this regard it is important to note that mesodermal components comprise the bulk of lung tissue and it is these cell populations that are responsible for the production of extracellular matrix, vascularization as well as other functions.

D'Ercole and Underwood (1980), and Adams, et al., (1983b), in concluding that somatomedin-C and MSA respectively were ubiquitous products of fetal rodent tissues, utilized immunological criteria which left open the possibility that the substance(s) produced by fetal lung fibroblasts was (were) distinct from somatomedin-C and MSA. It is likely that tissue-specific mitogens all share some structural homology which imparts commonality in antigenic determinants, but may differ from one another in amino acid composition. In lieu of sequencing lung mitogen(s), which would necessitate purification to homogeneity, evidence of structural distinctiveness can be obtained by techniques which exploit relative differences in amino acid composition. Techniques such as ion-exchange chromatography, sephadex gel filtration and analytical polyacrylamide gel electrophoresis can be applied to partially purified preparations of the
mitogen(s) and would obviate the need for large scale processing of culture media.

In order to determine the identity of the mesenchymal factor(s), it would first be necessary to initiate cultures consisting predominantly of fibroblasts, harvest the medium, and isolate and characterize the factor. From these studies it would be possible to determine if it was a somatomedin-like peptide and whether it was authentic somatomedin-C, as suggested by the studies of D'Ercole and Underwood (1980), MSA as suggested by Adams, et al., (1983a) or a distinct factor. Furthermore it would be of prime importance to determine the possible biological actions of the factor(s) in the developing lung.

There is evidence from Smith's (1981) studies that systemic hormones such as cortisol control the production of the factor(s). It can be hypothesized that systemic hormones orchestrate fetal development via the production of local tissue-distinct growth factors. It would be important to determine if the production of the local factor(s) was under systemic hormonal control.
MATERIALS AND METHODS

ISOLATION OF LUNG DERIVED PEPTIDES

Animals

Female Wistar rats (250-300gms) were mated and vaginal lavage samples were inspected for the presence of sperm. A sperm positive smear designated day 0 of fetal gestation. Animals were sacrificed at specific gestational ages and lung tissue was obtained from the fetuses for culturing.

Organ Explant Cultures

Fetal lung cells were obtained from day 19 fetuses. Each maternal rat was given an overdose of Nembutal (1% solution of sodium pentabarbital) which consisted of approximately 0.15 ml of Nembutal/kg body weight. Complete anesthesia, which usually took 15 minutes, was ascertained by absence of flexion of the lower limb when the foot was pinched. All dissections were performed in a stagnant air hood. After laparotomizing the fully anesthesized maternal rat through a midline incision, the entire pregnant uterus was immersed in cold, Ca++-Mg++ free sterile buffered saline (HBSS). An incision of the anti-mesometrial aspect of each uterine horn was made and the fetal-placental units were removed aseptically. Each fetus was dissected free from its amnion and the umbilical cord was severed. A midline incision was made in each fetus and the lung-heart
unit removed and immersed in cold HBSS containing antibiotics. The lungs were then minced into 0.25mm squares using a McIlwain Tissue Chopper in a stagnant hood. The tissue fragments were immersed in sterile HBSS in sterile 50 ml polystyrene Corning tubes and transferred to a SterilGard vertical laminar flow hood (Barker Co.) where all subsequent procedures were performed under sterile conditions. The tissue fragments in HBSS were shaken vigorously to remove macrophages, allowed to settle, and the loose cells were decanted. This procedure was repeated 3 times, after which a 0.25 g/dl collagenase solution in HBSS was added to partially digest the tissue. After 15 mins the digestion was stopped by adding HBSS containing 10% fetal bovine serum (FBS). The cells were pelleted by centrifugation and resuspended in culture medium consisting of a 1:1 mixture (v/v) of Ham's F-12 and Dulbecco's Modified Eagles Medium (DME) supplemented with gentamycin (5 mg/dl), penicillin (100 IU/ml), streptomycin (100 mcg/ml), fungizone (2.5 mcg/ml) and 10% FBS. The medium had previously been sterilized by passage through a 0.22 micron filter using a sterile Naglene filtering unit. The explants were plated in 75cm² or 150cm² Costar culture flasks with just enough medium to coat the flask bottom (8 mls and 16 mls respectively) and incubated at 37°C in humidified 95:5 air:carbon dioxide.

The explants were allowed to attach overnight. The
following morning they were washed with phosphate buffered saline (PBS) three times and were then incubated in serum-free culture medium (SFM) as described above. The medium was changed again 24 hours later to insure that all traces of FBS had been removed. All medium change procedures were performed under sterile conditions in the laminar flow hood. The SFM conditioned by these cells (CSFM) was collected at 3-day intervals. It was filtered through a nylon bolting cloth (41um mesh size), pooled, and kept at -4°C until processing.

PEPTIDE ISOLATION

Ion Exchange Chromatography

Batches of CSFM consisting of 0.3-1.0 liters were first adjusted to pH 6.8 before passing through a column (1.6 X 40cm; 75 ml bed volume) containing AG 50-1X8 cation exchange resin (Bio Rad) in the Na+ form. Before use the column was equilibrated with unbuffered 0.9% NaCl solution. The CSFM was then applied and the column was developed at room temperature by sequential treatment with 100 mls of 0.1M sodium bicarbonate solution adjusted with NaOH to pH 9.0 followed by a 0.1M ammonium hydroxide solution at pH 11. Eight milliliter fractions were collected. For each fraction the pH was monitored (Beckman Zeromatic pH Meter) as was the optical density at 280 nm (Beckman DU Spectrophotometer). The ammonium hydroxide fraction was neutralized with 2N HCl, dialysed overnight against acetic
acid in Spectra-Por 3 (MW cut off = 3,000) and subsequently lyophilized. The material was reconstituted in 50% (v/v) acetic acid. The suspension was then centrifuged for 2 minutes in a Beckman Microfuge B microcentrifuge and the supernatant collected and designated preparation pH 11.

**Sephadex Gel Filtration**

The reconstituted Fraction pH 11 in 50% acetic acid (HAc) was applied to a Sephadex G-50 or G-75 column (1.2 X 115cm) in a volume not exceeding 500μl (Moses, et al., 1980). The column was eluted with 1M HAc at room temperature, and 5 ml fractions were collected. Each aliquot was monitored spectrophotometrically at 280nm for protein content. The fractions were individually assayed for immunoactivity and individually lyophilized for bioassay in the chick embryo fibroblast assay (CEFA) (see Results p.39). When not individually bioassayed, the immunoreactive peaks were pooled, lyophilized and the activity of the pool was determined using the CEFA (see Biological Characterization section).

**Polyacrylamide Gel Electrophoresis**

Biologically active fractions of Sephadex G-75 were analyzed in a disc acrylamide electrophoresis system containing 15% acrylamide (Kodak) at pH 4.3. This method was modified from Reisfeld (1962). Disc acrylamide electrophoresis was run in glass tubes 75 X 5mm (inner
diameter) utilizing a Buchler Polyanalyst analytical apparatus (Searle). Prior to analysis the acrylamide solution was filtered using a 0.2μm filter. A 2.5% acrylamide spacer gel was used to concentrate the sample. The lyophilyzed samples were reconstituted using 25μl of 1M acetic acid, and after 1 hour 5μl of 0.1% methylene blue tracking dye in 1M acetic acid and 20μl glycerol was added. The chamber buffer consisted of 2.85 M B-alanine buffered with acetic acid at pH 4.5 and the samples were run at a constant current of 3 mA/gel with reverse polarity.

**Protein Determination**

Protein determinations were done by the method of Lowry, et al. (1951), or the Dye technique (BioRad). The optical density of each sample was determined on a Klett-Summerson photoelectric colorimeter, using a 660μm filter. Bovine serum albumin was used as the standard. When protein solutions were very dilute, aliquots were first lyophilized and reconstituted in small volumes.

**Radioimmunoassay**

The reagents for the somatomedin-C radioimmunoassay were supplied by Dr. Louis Underwood, University of North Carolina at Chapel Hill via the National Institutes of Health. The somatomedin reference standard used was human acromegalic serum purified by acid chromatography. Assays were carried out in 30mM sodium phosphate at pH 7.5
containing 0.25% bovine serum albumin (Sigma), 0.02% sodium azide, protamine sulfate, and 10mM EDTA. Antihuman Sm-C antiserum was diluted 1:4000 in assay buffer and the reaction initiated by adding 50μl to the unknown sample. The sample was incubated for 24 hours at 4°C. Trace ¹²⁵I-Sm-C was diluted to 5,000 cpm/50μl after treatment with ion-exchange resin (Bio-Rad Anion Exchange Resin AG 1-X8 200-400 mesh chloride form) to remove free iodine and 50μl was added to each sample tube. Incubation proceeded for another 24 hours at 4°C. Separation of bound from free counts was achieved by using a double antibody precipitation technique. Rabbit gamma globulin was diluted with assay buffer to a final concentration of 1mg/ml and 50μl added to the sample. Incubation continued overnight at 4°C. This is followed by adding 5μl (1 drop) of neat antirabbit gamma globulin. Precipitation was allowed to proceed at room temperature for 1 hour or overnight at 4°C. All reagents were added using micrometric pipettes (Gilson Pipettesman). The samples were centrifuged at 2000 RPM for 20mins at 4°C and the supernatant decanted. The samples were counted using a Beckman Gamma counter.

BIOLOGICAL CHARACTERIZATION

Chick Embryo Fibroblast Assay

Fractions at all stages of the isolation procedure were monitored for [³H]-thymidine incorporation into DNA
activity. Following the method of Dulak and Temin (1973a), tertiary cultures of nine day old chick embryo skin fibroblasts were plated in 24-well plastic clusters (Costar) and allowed to grow to confluence. The cells were serum starved for 24 hours before being exposed to the test medium. Labeled thymidine was added at least 1 hour after sample exposure and incubated for 18 hours. Cells were washed with cold buffered saline twice, protein precipitated with 0.2N perchloric acid and dissolved in 1N NaOH. Two aliquots were taken per well: one for counting using a Packard Tri-Carb liquid scintillation spectrometer, the other for protein determination employing the Lowry technique.

Rat Lung Fibroblast Assay

Cultures:

Both fetal and neonatal fibroblast cultures were established for experimentation. The procedure for dissection of the fetal tissues was identical to that described for initiating organ explant cultures. The neonatal rats were first decapitated, a transverse incision was made below the rib cage followed by a midline incision adjacent to the sternum, exposing the heart and lungs. For both fetal and neonatal rats, the lungs were removed, dissected free of visible elements of the tracheobronchial tree and then minced into 0.25mm blocks. The tissue fragments were treated as described above except that the
tissue was dispersed completely using an enzyme solution consisting of 100mg% collagenase (Sigma), 200mg% pronase (Calbiochem), 50mg% chymopapain (Sigma), 1mg% DNAase, 35 µ/ml elastase (Calbiochem) and 2% FBS in HBSS. The tissue was digested for approximately 15 minutes with intermittent pipetting to dissociate the cells. The suspension was passed through a 160um nylon bolting mesh and diluted with 10% FBS in HBSS, pelleted, resuspended in 10mg% DNAase in HBSS and incubated for 10-15 mins at 37°C. The cells were then pelleted, resuspended in DME culture medium with 10% FBS and plated in 24-well Costar multi-well clusters. Fibroblasts attach about 2 hours after plating and, for this reason, unattached cells were discarded after two hours, the medium replenished, and the fibroblasts were permitted to grow to confluency in 10% FBS.

Thymidine Incorporation:

Two separate protocols were used. For the first protocol cultures were treated exactly as for the chick embryo fibroblast assay, except these cultures were preincubated in SFM for 3 days before an experiment. Subsequently, the cells were incubated with the test hormone for a continuous 18 hour period. In the second protocol, cells were incubated with the test materials at specific concentrations and were subjected to a removal and replenishing of the medium at given concentrations so as to
minimize concentration changes that might occur due to endogenous production of bioactive substances. The medium of these cells was changed every 3 hours for a total of 12 hours at which time SFM containing labeled thymidine was added. The cells were incubated for an additional 16 hours, the incubation terminated by washing with cold buffered saline, precipitation with 0.2N perchloric acid, and dissolved in 200μl of 1N NaOH. An aliquot was used for counting, another for protein determination.

**Choline Bioassay**

The treatment of neonatal tissues used in obtaining these cultures was identical to that used for initiating rat lung fibroblast cultures up to the incubation with 10mg% DNAase. After this incubation the modified method of Keller and Ladda (1979) was used to initiate type-II cell cultures. The cells obtained from the digested tissue were pelleted, resuspended and subjected to discontinuous density gradient centrifugation using 10 and 20gm% Metrizamide in HBSS solutions. The cells at the 10-20% interphase were collected, washed, pelleted and, plated in Hams F-12 medium with 10% FBS. After two hours the loose cells which consisted mainly of type II cells were removed and replated in 24-well Costar clusters and allowed to attach overnight. Predominantly type II cell cultures were incubated in SFM for 24 hours, after which they were
incubated with or without hormones for the subsequent 48 hours. The incubation was stopped by washing the cells with cold HBSS and subsequent trypsinization for 8-10 mins at room temperature. The loose cells were transferred to 12X75 cm culture tubes and diluted 1:10 (v/v) with sterile saline. The cells were pelleted, the supernatant decanted, resuspended in saline, and repelleted to wash off excess label. After the supernatant was completely decanted 400 ul of distilled water was added to each tube and the cells were subsequently sonicated using a Heat Systems Cell Disruptor. To extract total lipids the method of Bligh and Dyer (1972) was employed. A 400 ul aliquot of the lipid-containing solvent phase was transferred to scintillation vials, evaporated in the vials, toluene scintillation cocktail was added and the samples counted.

STATISTICS

Statistical significance was determined by use of Student's T-test.
RESULTS
PARTIAL ISOLATION OF LUNG DERIVED MITOGEN

Primary organ explant cultures were used for harvesting purposes. It was found that fetal rat lung explants from gestational day 19 fetuses would attach readily and remain attached for up to 2 months in culture. The procedures used to isolate peptides from the conditioned serum free medium (CSFM) of fetal lung explants followed those used in the isolation of Multiplication Stimulating Activity (MSA) (Dulak and Temin, 1973a; Moses et al., 1980). The modified isolation scheme used in these studies is outlined on Figure 1.

Ion Exchange Chromatography

Conditioned serum-free medium from nineteen-day old fetal rat lung organ cultures adjusted to pH 6.8 was chromatographed using a cation exchange resin (Figure 2). Adsorbed cationic species were eluted off the column by successive washings with 100mls of 0.9M NaCl, 0.1M NaHCO₃ at pH 9 and, 0.1M NH₄OH at pH 11. Each fraction was also monitored spectrophotometrically for protein and for immunoactivity using the somatomedin-C radioimmunoassay. There were two immunoreactive peaks which corresponded to protein peaks associated with the pH 9 and the pH 11 washings. This was evidence for the presence of at least two immunoreactive protein species. The fractions
FIGURE 1. ISOLATION SCHEME

CSFM

Ion-exchange Chromatography
AG 50W-X8, Na⁺ form

Unretained

- Anion exchange

NaHCO₃ elution

- HCl elution
  - pH 2

NH₄OH elution

- pH 11
  - neutralize
  - lyophilize

HAc extraction

Sephadex G-75 Gel Filtration

- pH 11-I 11-II 11-III
FIGURE 2. CATION EXCHANGE CHROMATOGRAPHY OF CONDITIONED SERUM-FREE MEDIUM (CSFM). Adsorbed cationic peptides are washed off the column by successive washings with NaHCO₃ at pH 9 and NH₄OH at pH 11 (top) and the pH of the eluate was monitored (·······). Fractions collected (8mls/fraction) were numbered 1-50. The protein content of each fraction was monitored spectrophotometrically at 280 nanometers (●●●●●). Each fraction was monitored simultaneously for immunoactivity using the somatomedin-C radioimmunoassay (○○○○○). The fractions corresponding to the two peaks were pooled and were respectively designated the pH 9 and pH 11 preparations.
corresponding to the two peaks were pooled and designated respectively the pH 9 and pH 11 preparations. The material was neutralized with 2N HCl and was dialyzed overnight against 2% acetic acid at room temperature and subsequently lyophilized. The results of the chromatography demonstrate that the majority of the mitogenic substance elutes off the column in the pH 11 effluent.

Furthermore, this isolation protocol was monitored for purification and recovery using the chick embryonic fibroblast assay (CEFA). The effluent corresponding to the pH 9 and pH 11 peaks, as well as the unretained, were dialysed, lyophilized, reconstituted and tested for bioactivity. The specific activity of each preparation was monitored and was defined in terms of $^3$H-thymidine incorporation where one unit of mitogenic activity is the amount of label incorporation elicited by 1mg/ml of fetal bovine serum. It was found that the unretained fraction contained only a small amount of mitogenic activity even after processing via anion exchange resin. This amounted to 7 units/liter as compared with 63.6 and 244.9 units/liter for the pH 9 and pH 11 respectively. The ammonium hydroxide effluent, the pH 11 preparation, contained the majority of both the biologically active, as well as the immunologically active substance (Table 1.).
TABLE 1: ISOLATION OF MITOGEN FROM CELL-CONDITIONED SERUM-FREE MEDIUM (CSFM)
Mitogenic activity was assessed by the chick embryo fibroblast assay (CEFA). One unit of activity is equivalent to the mitogenic potency of 1 mg/ml fetal bovine serum. Protein concentration was measured by the Lowry technique.

<table>
<thead>
<tr>
<th>Isolation step</th>
<th>Preparation</th>
<th>Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Recovery %</th>
<th>Specific Activity (units/mg Pr)</th>
<th>Enrichment (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>CSFM</td>
<td>3,684</td>
<td>4,495</td>
<td>100</td>
<td>1.22</td>
<td>1</td>
</tr>
<tr>
<td>AG 50W-X8</td>
<td>Unretained:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 2</td>
<td>13.1</td>
<td>70</td>
<td>2</td>
<td>5.3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Retained:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 9</td>
<td>9.8</td>
<td>636</td>
<td>14</td>
<td>64.9</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>pH 11</td>
<td>19.1</td>
<td>2,449</td>
<td>54</td>
<td>128.2</td>
<td>105</td>
</tr>
</tbody>
</table>
Gel Filtration

To determine if the pH 11 preparation contained more than one active peptide species, Sephadex gel filtration was performed after reconstituting the pH 11 preparation with 50% (v/v) acetic acid. This extraction in acetic acid was done to dissociate the peptide from binding protein and to reduce aggregation. The chromatographic profile, seen in Figure 3, shows the fractions collected and tested simultaneously for protein content, immunoactivity and, bioactivity. The majority of the protein eluted near the void volume (32-44mls) had immunological activity but no biological activity. This high molecular weight component probably represents binding protein. This material was additionally shown to displace antibody in the Sm-C radioimmunoassay (Sm-C RIA). On the chromatographic profile, two immunoreactive peaks were demonstrated: peak II (48-56mls), having a $K_a$ value of 0.31 (Range: 0.23-0.38), and peak III (60-72mls), having a $K_a$ value of 0.54 (Range: 0.46-0.69). The last of these was found to contain the majority of the biological as well as immunological activity. The fractions corresponding to this peak were pooled and designated the pH II-III preparation. Figure 3 also demonstrates that the molecular weight of II-III is between 13,000 and 6,000 daltons.
FIGURE 3. SEPHADEX G-75 CHROMATOGRAPHY OF THE pH 11 PREPARATION. Fractions were collected in 4 ml aliquots. Each fraction was monitored simultaneously for cross-reactivity in the somatomedin-C radioimmunoassay and mitogenic activity in the chick embryo fibroblast assay (CEFA). The results are expressed as percent inhibition of binding (-----) and percent change from the control (▲-----▲), respectively. The protein content was measured using the Dye technique. The column was calibrated using cytochrome-C (13 kilodaltons), insulin (6 kilodaltons), and blue dextran. The fractions corresponding to the three mitogenic peaks were pooled and designated pH 11-I, 11-II and 11-III.
Dose Response Studies

The somatomedin-C radioimmunoassay and the CEFA were used to monitor overall enrichment of the immunoreactivity and bioactivity at each step of the isolation procedure. The competitive dose-response curves are shown in Figure 4. The ordinate shows specific binding of labeled Sm-C and the abscissa shows the unlabeled competitor in absolute protein per tube. This graph demonstrates an enrichment of three orders of magnitude of the immunoreactive material from the starting material to the pH II-III preparation. Acid chromatographed human acromegalic serum supplied by the National Institutes of Health (NIH) is an enriched source of Sm-C. This preparation as well as commercially obtained Multiplication Stimulating Activity (MSA) (Collaborative Research) were also assayed. As can be seen from Figure 4, the pH II-III curve lies to the left of the NIH/Sm-C curve. It is not parallel to the competitive binding curve of the NIH/Sm-C standard while it does appear to parallel that of the MSA suggesting these may be antigenically similar.

Dose response curves were similarly constructed to monitor bioactivity using the CEFA. The results are seen in Figure 5. Incorporation of tritiated thymidine is plotted on the ordinate expressed as percent increase of control. Protein concentration is seen on the abscissa. A similar enrichment of the mitogenic material could be traced and was shown to increase at each successive purification
FIGURE 4. MEASUREMENT OF IMMUNOREACTIVITY OF LUNG DERIVED FACTORS BY THE SOMATOMEDIN-C RADIOIMMUNOASSAY. A single batch of conditioned serum-free medium (CFSM) was processed according to the isolation scheme outlined in Figure 1. Preparations derived from sequential isolation steps were dialysed, lyophilized, reconstituted in 10mM HCl and were assayed for immunoactivity. Protein was determined by the method of Lowry, et al., (1951). Multiplication stimulating activity (MSA) was purchased from Collaborative Research and a somatomedin-C enriched preparation of human acromegalic serum obtained from the National Institutes of Health were used as standards. The data are expressed as relative specific binding versus total protein added per assay tube.
FIGURE 5. MEASUREMENT OF BIOACTIVITY OF LUNG DERIVED FACTORS IN THE CHICK EMBRYO FIBROBLAST ASSAY (CEFA).

A single batch of conditioned serum-free medium (CSFM) was processed according to the isolation scheme outlined in Figure 1. Preparation derived from sequential isolation steps were dialysed, lyophilized, reconstituted in 10mM HCl and analysed for $^3$H-thymidine incorporation into DNA in CEFA. Protein was measured by the method of Lowry, et al., (1951). Multiplication stimulating activity (MSA), purchased from Collaborative Research, was used as a standard to monitor cell responsiveness. The data are expressed in percent change $^3$H-thymidine incorporation from control versus protein concentration per assay well.
CHICK EMBRYO FIBROBLAST ASSAY

THYMIDINE INCORPORATION % INCREASE

PROTEIN (ng/ml)

-msa

- 11-III

- CSFM

- 11

0 100 1000 10000
stage. Collaborative Research MSA (CR-MSA) exhibited a
greater mitogenic potency than the pH 11-III preparation in
the CEFA system (Figure 5).

**Analytical Disc Acrylamide Gel Electrophoresis (PAGE)**

In order to evaluate the homogeneity of the pH 11-III
preparation it was electrophoresed using 15% acrylamide
gels in acetic acid at buffer pH 4.5. Figure 6 shows the
results of one experiment where the pH 11-II and the pH 11-
III preparations were electrophoresed on duplicate gels.
The gels were either stained with Coomassie Blue or cut
into 1mm slices. The slices were eluted overnight in
Earle's buffered saline containing 0.5% bovine serum
albumin. The eluate was subsequently tested for
immunoactivity. The electrophoretic pattern for the pH 11-
II preparation contained several stainable bands with $R_f$
values of 0.15, 0.22, 0.29, 0.42 and, 0.61. The major
immunoreactive peaks appeared at $R_f$ value of 0.33 and a
minor component at 0.26. These peaks did not correspond to
the stainable bands. Electrophoresis of the pH 11-III
preparation revealed only one stainable band at $R_f$ value
0.70 which did not correspond to the major immunoreactive
peak at 0.59. This demonstrated that the Sephadex peaks
11-II and 11-III were composed of two distinct populations
of peptides.
FIGURE 6. ELECTROPHORETIC PROFILES OF II-II AND II-III. Pools of II-II and II-III were obtained after Sephadex gel filtration. The samples were electrophoresed on 15% acrylamide gels as described in Materials and Methods. Two gels were simultaneously electrophoresed for each of the two preparations. One of the pair was stained for protein with Coomasie Blue. The other gel was sliced into 1 mm slices and eluted overnight in Earl's buffered saline solution containing 0.5% bovine serum albumin. The elute was then assayed using the somatomedin-C radioimmunoassay. The results are plotted as percent inhibition of binding versus electrophoretic mobility expressed in Rf units.
To determine whether the immunoreactive peak corresponded to the mitogenic substance, a pH II-III preparation was run and simultaneously tested for immunoactivity and mitogenic activity (Fig 7). A major immunoreactive peak emerged again at $R_f$ value 0.59. Testing of the slices for mitogenic activity revealed that a number of mitogenic species were present. The major peaks at $R_f$ values of 0.59 and 0.53. The lesser peaks found at 0.47, 0.65 and, 0.33 did not cross react with the somatomedin-C RIA. These studies showed that II-III was a heterogeneous preparation containing several mitogenic species, at least one of which did not appear to cross react with the Sm-C RIA system.

**Acid PAGE of $^{125}$I-Somatomedin-C**

The electrophoretic mobility of Sm-C had been previously reported to be 0.41 +/- 0.01 using 18% acrylamide gels in the same buffer system (Svoboda, et al., 1980. Figure 8 shows the electrophoretic profile for $^{125}$I-Somatomedin-C in this acid PAGE system. Two peaks are seen; one at $R_f$ value 0.29 the second at $R_f$ value 0.53 +/- 0.02 (n=3). The first peak is probably due to aggregated damaged label and the second peak represents authentic Sm-C. Thus the major component at $R_f$ 0.59 of the II-III preparation is not authentic Sm-C.
FIGURE 7. BIOASSAY AND RADIOIMMUNOASSAY OF II-III ELECTROPHORETIC PROFILE. The II-III preparation was electrophoresed as described in Materials and Methods and the gel sliced in 1mm sections. After overnight elution of the slices in Earl's buffered saline solution containing 0.5% bovine serum albumin, the eluate was simultaneously assayed for mitogenic activity using $^3$H-thymidine incorporation in the chick embryo fibroblast assay (CEFA) and immunoactivity using the somatomedin-C radioimmunoassay. The data are expressed in counts per minutes ($\Delta \cdots \Delta$) and percent inhibition of binding (•------•), respectively, versus electrophoretic mobility in $R_f$ units.
% INHIBITION of BINDING
FIGURE 8. ELECTROPHORETIC PROFILE OF $^{125}$I-SOMATOMEDIN-C. $10^3$ cpm of $^{125}$I-somatomedin-C were electrophoresed as described in Materials and Methods. The gel was sliced into 1mm slices, and counted. The data are expressed in counts per minute versus electrophoretic mobility in $R_f$ units.
BIOLOGICAL CHARACTERIZATION

In order to study the potential paracrine function of the mitogens contained in the II-III preparation, the following series of experiments were executed.

**[3H]-Thymidine Experiments**

As shown by the dose response study using chick embryo fibroblast, II-III did stimulate mitosis in a dose dependent manner (Figure 5). In order to test the mitogenic potency of this preparation on homologous rat cells, rat lung fibroblasts were incubated with [3H]-thymidine in the presence and absence of the pH II-III preparation for a total of 18 hours. Figure 9 shows that preparation II-III stimulated mitosis in postnatal day 4 rat lung fibroblasts in a dose-dependent manner. As demonstrated here, however, fetal fibroblasts were producing the mitogenic peptides contained in the II-III preparation, therefore a procedure had to be devised to look at the mitogenic effects controlling for endogenous production of factors.

To examine the mitogenic effects of II-III on the cells which produce it, monolayer cultures of day 19 fibroblasts were incubated with or without hormone and the medium was changed every 3 hours for 12 hours. A set of control wells received no hormone and no medium change during the 12 hour period. Subsequent to the 12 hour period, the test medium was removed from all wells, fresh
FIGURE 9. EFFECT OF I-III ON $^{3}$H-THYMIDINE INCORPORATION IN POSTNATAL RAT LUNG FIBROBLASTS. Mitotically quiescent confluent cultures of lung fibroblasts from four-day old rat pups were exposed to varying concentrations of I-III in the presence of 0.5 uCi/ml $^{3}$H-thymidine for 18 hours. Results are expressed relative to thymidine incorporation rates in cultures not exposed to I-III. Fetal bovine serum (1mg/ml) served as a positive control.

* $P < 0.05$

** $P < 0.01$
THYMIDINE INCORPORATION

% Change

\[ \text{SH11 III (ng/ml)} \]

S.E.

n=2

FBS
medium containing $^{3}$H-thymidine was added and incubated for an additional 12 hours. Figure 10 shows the various treatment groups labeled on the abscissa while on the ordinate is plotted thymidine incorporation as percent change from the no medium change control. The results indicate that under these conditions fetal cells were not responsive to the mitogens in the II-III preparation, although they were responsive to fetal bovine serum as well as MSA purchased from Collaborative Research.

Figure 11 shows the results for monolayers of day 4 neonatal cells using the same procedure as well as a control treatment group which received no medium change during the first 12 hours. Thymidine incorporation was significantly stimulated at the highest dose of II-III (1μg/ml) in the no medium change group, although the same dose failed to stimulate mitosis in cells whose medium was continually replenished.

One way of explaining these seemingly contradictory results was the possibility these cells were producing other factors which were necessary for the expression of the mitogen. These factors were perhaps being depleted during the change of medium protocol. A more comprehensive study was done which involved comparing II-III with a dialyzed and lyophilised sample of CSFM. Two standards were used: FBS and a preparation of MSA (BRL-MSA), which has pure progression activity and was freshly purified from
FIGURE 10. EFFECT OF 11-III ON $^3$H-THYMIDINE INCORPORATION INTO FETAL LUNG FIBROBLASTS. Confluent, mitotically quiescent cultures of day 19 fetal rat lung fibroblasts were subjected to four medium changes at 3 hour intervals in the presence or absence of 11-III (0, 100, 1000 ng/ml) to eliminate interference by endogenously produced mitogens. Results are expressed as percent change from the zero dose, no medium change control.

- Experiment A, in which fetal bovine serum (1mg/ml) served a positive control.

- Experiment B, in which MSA (Collaborative Research) served as a positive control.

* P< 0.05

** P< 0.01
THYMIDINE INCORPORATION

% Change

I = X±SE of 3 replicates

PH 11

FBS

MSA

---

---

---

---

FIGURE 11. EFFECT OF 11-III $^3$H-THYMIDINE INCORPORATION INTO POST-NATAL DAY 4 RAT LUNG FIBROBLASTS: EVIDENCE FOR ENDOGENOUSLY PRODUCED GROWTH FACTOR. Comparison of 2 experimental protocols: A) exposure to 11-III for a continuous 18 hours. B) exposure to 11-III with replenishment of medium every 3 hours. Results are expressed as counts per minute versus 11-III concentration (1, 10, 100, 1000 ng/ml) for 2 protocols. Fetal bovine serum (1 mg/ml) was used as a positive control to monitor cell responsiveness.

$T = X \pm S.E.$ of 2 replicates.

* $P < 0.05$
** $P < 0.01$
the CSFM of Buffalo rat liver cells and processed by the method of Moses, *et al.* (1980). Figure 12 demonstrates that neither BRL-MSA nor II-III alone stimulated proliferation of day 19 fetal rat lung fibroblasts, while the starting material induced a dose dependent stimulation.

To confirm the mitogenic role of II-III in fetal lung fibroblast and further test the hypothesis that the missing component was a competence factor, the cells were pre-exposed to a known competence factor, FGF, for only 3 hours and the experiment proceeded as before. The results, seen in Figure 13, clearly show II-III to be mitogenic at a dose of 1000 ng/ml in cells which were rendered competent by FGF.

**Dose Response Studies in the Presence of EGF**

Epidermal growth factor (EGF) has been implicated to have a maturational and growth promoting role in the fetal lung (Sundell, *et al.*, 1980) and receptors to EGF have been detected in high concentrations in fetal mouse tissues (Nexo, *et al.*, 1980). To determine if the II-III mitogen was exerting its effects via the EGF receptor system, dose response study was conducted using cultures of fibroblasts from postnatal day 4 rat lungs in the presence and absence of EGF. As can be seen in Figure 14, the presence of EGF resulted in an additive mitogenic response, shifting the entire dose response curve upward. This
FIGURE 12. EFFECT OF II-III ON $^3$H-THYMIDINE INCORPORATION IN FETAL DAY 19 RAT LUNG FIBROBLASTS: EVIDENCE FOR ENDOGENOUSLY PRODUCED COMPETENCE ACTIVITY. Fibroblasts were exposed to various concentrations of II-III, buffalo rat liver cell-derived multiplication stimulating activity (BRL-MSA) and unprocessed conditioned serum-free medium (CSFM). The medium containing the appropriate test dose was replenished every 3 hours for a total exposure time of 12 hours. The data are expressed relative to thymidine incorporation rates in control cultures. FBS was used to monitor responsiveness of the cells.

* P < 0.05
** P < 0.01
FIGURE 13. FETAL LUNG FIBROBLASTS REQUIRE EXPOSURE TO COMPETENCE ACTIVITY FOR RESPONSIVENESS TO II-III: EVIDENCE THAT II-III IS A PURE PROGRESSION FACTOR. Confluent, quiescent cultures of fetal day 19 lung fibroblasts were pre-treated with FGF at a concentration of 300ng/ml for 3 hours after which time the cells were exposed to II-III at a concentration of 1μg/ml, or buffalo rat liver cell-derived multiplication stimulating activity (BRL-III) at a concentration of 1μg/ml. Fetal bovine serum (FBS) served as a positive control to monitor cell responsiveness. The results are expressed relative to 3H-thymidine incorporation rates in control cultures that were not exposed to growth factors.

* P < 0.05
** P < 0.01
FIGURE 14. MITOGENIC RESPONSE OF POST-NATAL DAY 4 RAT LUNG FIBROBLAST TO II-III IN THE PRESENCE OF EPIDERMAL GROWTH FACTOR (EGF). Post-natal day 4 monolayers of rat lung fibroblasts were incubated in serum free medium for at least 24 hours. Cultures were exposed to II-III alone or to II-III plus EGF for a total of 20 hours. The data are expressed in dpm as a function of II-III concentration.

* P > 0.01
suggests that II-III and EGF are acting via separate receptor systems to stimulate proliferation.

[14C]-Choline Incorporation Experiments

These experiments were carried out to determine if the pH II-III preparation contained a substance that could influence the incorporation of choline into pulmonary surfactant, the surface active material secreted by the alveolar type II cells. A surfactant stimulating factor having an apparent molecular weight between 5000-7000 daltons had been reported by Smith (1979).

[14C]-Choline Incorporation using Mixed Cell Cultures

Mixed cell cultures from fetal and post-natal lungs were initiated. Figures 15 and 16 contrast rat lung fibroblast cultures with mixed cell cultures prepared simultaneously from the same cell suspension. The cells in these photomicrographs were obtained from the lungs of 15 day old neonatal rats and were taken at the light microscope level after two days in culture. It can be seen that the mixed cultures (Figure 16) contain cells morphologically distinct from the fibroblasts seen in monolayers which are characterized by their spindle shapes (Figure 15). It should be noted that the mixed cultures did contain more than one distinguishable cell type as can be seen in Figure 17 A and B. Mixed cell cultures from postnatal day 15 lungs were composed of approximately 70%
FIGURE 15. PHOTOMICROGRAPHS OF RAT LUNG FIBROBLAST CULTURES. Both fibroblast and mixed cell cultures were obtained from the same tissue pool of post-natal day 15 rat lung and were plated simultaneously. (A) Rat lung fibroblast monolayer after 48 hours in culture. 100X magnification. The swirl-like pattern of growth typical of fibroblast cultures is seen here. (B) Rat lung fibroblast monolayer after 48 hours in culture. 200X magnification. The characteristic spindle shape of the fibroblasts can be distinguished in the lower left of the photograph.
FIGURE 16. PHOTOMICROGRAPHS OF MIXED RAT LUNG CELL CULTURES. As explained in the previous figure, both fibroblast and mixed cell cultures were obtained from the same tissue pool of postnatal day 15 rat lung and were plated simultaneously. (A) Mixed cell culture after 48 hours in culture. 100X magnification. (B) Mixed cell culture after 48 hours. 200X magnification. These cultures have round cells with large central nuclei, contrasting sharply with the fibroblast cultures.
FIGURE 17. PHOTOMICROGRAPHS OF MIXED CELL CULTURES. (A) At a magnification of 400X vacuoles of various sizes become visible in the cytoplasm of many of the rounded epithelioid-type cells. (B) In the same culture cells resembling neither fibroblasts nor epithelial cells can be found. 400X magnification.
type-II cells as determined by transmission electron microscopy.

To determine the responsiveness of these cultures to known stimulators of surfactant in mixed cell cultures, a dose response study was done using cortisol. It was found that surfactant stimulation was significantly increased at a concentration of $10^{-6}$ molar cortisol in day 19 mixed cell cultures (Figure 18).

Surfactant synthesis in these cultures, however, was not stimulated by the 11-III preparation. Clearly seen in Figure 19 is the fact that, though the cultures were responsive to cortisol, exposure to 11-III at a concentration of 1000ug/ml did not stimulate the incorporation of $^{14}$C-choline into cell lipids in cultures isolated at various ages. Simultaneous exposure to both cortisol and 11-III resulted in either no effect or a response intermediate in magnitude between cortisol and 11-III alone.

$^{14}$C-Choline Incorporation Studies using A549 Cultures

Further investigation of 11-III's potential effects on type-II cell function proceeded using the A549 cloned type-II cell line.

When the cells were exposed for 24 hours, 11-III at 1ug/ml did not stimulate but rather depressed choline incorporation in A549 cells both alone and in the presence of cortisol (Figure 20). Also seen in these results is
FIGURE 18. EFFECT OF CORTISOL ON PHOSPHOLIPID SYNTHESIS IN PRIMARY MIXED LUNG CELL CULTURES. Cell cultures were incubated in serum free medium for at least 18 hours prior to hormone exposure. Subsequently, cultures were incubated with cortisol at varying concentrations (10⁻¹⁰, 10⁻⁸, 10⁻⁶) in the presence of ¹⁴C-choline. Total incubation time was 48 hours at which time the cells were trypsinized off the cluster wells and transferred to glass tubes. Cellular lipid was extracted as described in Materials and Methods. The results are expressed relative to choline incorporation in the control cultures.

** P > 0.01
FIGURE 19. EFFECTS OF 11-III ON PHOSPHOLIPID SYNTHESIS IN PRIMARY LUNG CELL CULTURES. Cells were isolated from rat lungs of various ages: fetal day 19 and 20, as well as postnatal day 15. Cultures were incubated in serum free medium for at least 18 hours prior to hormone exposure. Cells were subsequently incubated with 11-III (1ug/ml), alone or in the presence of cortisol (10^-8/10^-8). Cortisol alone was used to monitor cell responsiveness.

* P > 0.05
** P > 0.01
FIGURE 20. PHOSPHOLIPID SYNTHESIS IN THE A549 CELL LINE: EFFECTS OF CORTISOL AND 11-III AFTER 24 HOURS OF EXPOSURE. A549 cells were exposed to 11-III (100, 1000 ng/ml), alone, or in the presence of cortisol (10^-10, 10^-8, 10^-6 M). The assay was incubated with the hormones for 24 hours. The results are expressed as dpm per ug protein in each assay well versus hormone concentration.
of cortisol (Figure 20). Also seen in these results is the fact that cortisol did not affect choline incorporation at any of the concentrations tested. In addition, when the hormone exposure period was extended to 48 hours, no change for any test treatments was seen (Figure 21). Analysis of the A549 cell conditioned medium showed no change of lipid extractable $^{14}$C-choline in the medium.

**II-III is Distinct from Surfactant Stimulating Factor**

Figure 22 shows the chromatographic profile for unprocessed CSFM passed through Sephadex G-75. The effluent was collected in 4 ml aliquots and numbered 1 through 24. The eluates were simultaneously assayed to measure immunoactivity using the somatomedin-C RIA (plate A, Figure 22), $^3$H-thymidine incorporation into DNA in two culture systems (i) the chick embryonic fibroblast assay (plate B), (ii) mixed type-II cell culture (plate C), and choline incorporation in mixed cell cultures (plate D). The results clearly demonstrate the presence of 2 bioactive substances, both derived from fetal lung fibroblasts. There was a large choline stimulating peak which eluted at a $K_{av}$ value of 0.22, having a molecular weight of approximately 13,000 daltons (plate D). This substance also stimulates proliferation in the mixed cell cultures (plate C). It is distinct from the immunoreactive and, CEFA- stimulating, II-III peak (A, B). Interestingly,
these peptides had no mitogenic activity in the mixed type-II cell cultures (comparison of A and B vs. C).
FIGURE 21. PHOSPHOLIPID SYNTHESIS IN THE A549 CELL LINE: EFFECT OF CORTISOL AND 11-III AFTER 48 HOURS OF EXPOSURE. A549 cells were exposed to 11-III (100-1000 ng/ml) alone or in the presence of cortisol (10^-10, 10^-8, 10^-6 M). The assay was incubated with the hormones for 48 hours. The results are expressed as dpm per ug protein in each assay well versus hormone concentration.
FIGURE 22. SEPHADEX G-75 CHROMATOGRAPHY OF CONDITIONED SERUM-FREE MEDIUM (CSFM): EVIDENCE FOR A SURFACTANT STIMULATING FACTOR DISTINCT FROM II-III. Fractions were eluted in 1M acetic acid and collected in 4 ml aliquots, numbered on the abscissa. Each fraction was monitored simultaneously for immunoactivity in the somatomedin-C radioimmunoassay (plate A), mitogenic activity in the chick embryo fibroblast assay (CEFA) (plate B), mitogenic activity in the mixed cell cultures (plate C), and $^{14}$C-choline incorporation into phospholipids in mixed cell cultures (plate D). The column was calibrated using cytochrome-C (13,000 daltons), insulin (6,000 daltons), and blue dextran.

Plate A: Immunoactivity from the collected fractions was assayed directly and the results are expressed in uU. The $K_{av}$ value of the peak is 0.50.

Plate B: Individual fractions were lyophilized, reconstituted (10mM HCl), and bioassayed. The CEFA results are expressed in mU of mitogenic activity. (One unit of activity = $^3$H-thymidine incorporation elicited by 1mg/ml of fetal bovine serum.)

Plates C & D: Mixed cell cultures were exposed to individual reconstituted fractions in the presence of $^3$H-thymidine and $^{14}$C-choline. Results are expressed relative to incorporation rates in control cultures. The $K_{av}$ value of the peaks is 0.23 for both mitogenic and surfactant stimulating activity.
DISCUSSION

PARTIAL ISOLATION OF LUNG GROWTH FACTORS

Our present understanding of growth factors results from a convergence of knowledge from 3 different areas of research on (1) the mediators of the growth-promoting actions of GH, (2) the identity of serum factors that stimulate cellular multiplication in vitro and (3) the purification and characterization of factors produced in vitro by specific cell populations. To date, growth factors which have been chemically identified have been shown to exhibit significant structural homologies across evolutionary lines. This provides support for the generalization that the MSA family and the somatomedin family of peptides belong to a class of mitogenic peptides which certain mammalian tissues are capable of producing in response to somatomammotrophins (growth hormone, prolactin, placental lactogen). Along the same lines, present evidence also attests to the concept of species specific families of growth factors.

Organogenesis during embryonal development is presently perceived as a process which is characterized by differential growth and maturation of different organ systems. This concept would argue against the pivotal role of non-site specific mitogens. The amply documented site-specificity of nerve growth factor production would attest to the belief that tissue-specific growth and
maturational factors must exist as important regulators in development.

Evidence brought forth by Alescio and Piperno (1967) and Smith (1979, 1981) support the theory that lung development proceeds under the inductive influence of locally produced peptide factors. Their studies, coupled with the fact that the fetal lung receives only a small fraction of the cardiac output and is considered a poorly perfused organ while being competitive in growth rate with other, better perfused tissues, provide strong argument for the existence of locally produced mitogen(s) in the fetal lung. The experiments described here were performed to determine whether fetal lung fibroblasts produce local peptide factors which might be playing a major role in fetal lung growth and maturation.

Preliminary results indicated that the conditioned medium of cultured lung fibroblasts did contain growth factors, as it was capable of stimulating mitosis in embryonic chick fibroblasts. The existence of these factors had been hypothesized by Alescio and Piperno (1967) but had not been systematically researched. The present studies focused on the in situ production of mesenchymal lung growth factors, their partial biochemical isolation and characterization, and their biological characterization.

Dulak and Temin (1973a, 1973b) first isolated a
family of low molecular-weight, mitogenic polypeptides from serum-free culture medium conditioned by a cloned Buffalo rat liver cell line. They subsequently named this family of liver-derived peptides "multiplication stimulating activity", or MSA. Using more sensitive biochemical techniques, Rechler, et al. (1980), distinguished seven biologically active components in the BRL-MSA family of growth factors. In the current experiments an isolation scheme similar to theirs was employed, using an initial cation exchange chromatography followed by gel filtration. The dose response studies employed to monitor successive isolation steps clearly demonstrated the effectiveness of these techniques in successively purifying the mitogen.

It is interesting to compare the present results for the lung derived CSFM to the isolation results of the liver derived CSFM. The percent recovery of mitogenic activity after ion exchange chromatography was 54% in the pH 11 preparation and 14% for the pH 9 effluent as determined by bioassay (Table 1). Dulak and Temin (1973a) report 58% and less than 2%, respectively, for this stage in their isolation procedures. Thus, recovery values for the mitogenic material using this technique were similar.

Moses, et al., (1980) found three bioactive peaks on gel filtration in Sephadex G-75 of the chromatographed BRL CSFM. The $K_{av}$ values were 0.18 (range: 0.13-0.22), 0.36
(range: 0.26-0.45) and, 0.56 (range: 0.48-0.64) for MSA-I, -II, -III, respectively. MSA peak III from G-75 was found to be composed of three species, one of which (MSA III-2) was purified to homogeneity and found to have a molecular weight of 7100 daltons (Moses, et al., 1980). The fetal lung fibroblast material revealed only two bioactive peaks on gel filtration having $K_a$ values of 0.50 (range: 0.44-0.56), and 0.72 (range: 0.61-0.83) for pH II-II and pH 11-III respectively (Fig. 3). Thus, the peptides which compose the pH 11-III fraction are in a lower molecular weight range than MSA III.

Using identical gel filtering techniques to chromatograph lyophilized lung CSFM, Smith (1979) reported a $K_a$ value of 0.45 for his fibroblast pneumocyte factor. It should be noted that the biological activity monitored in his studies was radiolabeled choline incorporation into phosphatidylcholine.

Analytical disc acrylamide electrophoresis of material from peak II-III demonstrated that we were dealing with at least 4 molecular species, two of which did cross react in the Sm-C RIA. The major component having an electrophoretic mobility of 0.59 (+/- 0.02) exhibited both mitogenic as well as immunoreactive properties (Fig. 7). Comparison of the electrophoretic migration of the II-III peptides with the mobility of radiolabeled Sm-C revealed that the major peptide at 0.59 was not Sm-C ($R_f = 0.53$)
+/-0.02). It should be noted that the Sm-C RIA system employed in these studies does exhibit crossreactivity with other growth factors. Specifically, it exhibits a 1.2% crossreactivity with MSA III-2 (Van Wyk, et al., 1980). In addition, rat IGF-I is 15% as reactive as human IGF-I in this system (Daughaday, et al., 1982). These results indicate that we are dealing with a member of the family of growth factors which has structural homologies with insulin but which is distinct from Sm-C.

BIOLOGICAL CHARACTERIZATION

The chick fibroblast assay is the standard bioassay commonly used in the detection of growth factors. The II-III preparation clearly did stimulate mitosis in a dose dependent manner using this system (Fig 5). Monolayer cultures of fetal rat lung fibroblasts, however, failed to respond when incubated with II-III alone (Figure 10). In these latter studies the endogenous production of II-III was controlled for by replenishing the medium every three hours during the hormone incubation period, a total of 12 hours. This protocol had the effect of abolishing the positive mitogenic response to the II-III preparation in post-natal rat lung fibroblasts (Figure 11). These experiments, coupled with a further study (Figure 12) demonstrating significant stimulation of mitosis by the unprocessed conditioned medium, but not in the presence of
progression factor alone, provided evidence for the existence of other endogenously produced substances necessary for the expression of mitosis in these mesodermal cells. Studies using human fibroblasts have demonstrated that mammalian cells do not respond to preparations of pure progression factors (Moses, et al. 1978, Antoniades, et al., 1975). Avian cells differ in that they do not require competence factors for proliferation. This would explain the mitogenicity of II-III in the chick fibroblast assay, and the lack of response in the rat lung fibroblast cultures. Figure 13 shows that with transient exposure to FGF, a competence factor, the rat lung fibroblasts did respond to II-III. It would thus appear that II-III is an important autocrine factor during development. The significance of such an autocrine mesodermal factor is clear when one considers that the bulk of lung tissue, and the principal fate of this organ, resides in its mesodermal components (smooth muscle cells, endothelial cells, and fibroblasts). In addition to allowing for vascularization and the regulation of regional blood flow, these are the cell populations that produce the extracellular matrix upon which the epithelium resides. During recent years, substantial evidence has accumulated which supports the hypothesis that extracellular materials direct differential cell behavior during morphogenesis. Studies indicate that the substrate upon which a cell rests
can dictate its shape and may play a role in a differential response to various growth factors (Wessells, 1964; Gospodarowicz, et al., 1976a).

In addition to making clear the fact that II-III exhibits pure progression factor activity, the rat lung fibroblast data also suggest the presence of an endogenously produced competence factor (Fig. 12). Comparison of the medium change protocol versus the no change protocol (Fig 11) supports this hypothesis. In addition, it was shown that exposure to lyophilized starting material, as well as transient exposure to the known competence factor, FGF, prior to II-III exposure, proved to stimulate cell division (Figures 12 and 13). These data suggest that the starting CSFM contains a competence-endowing factor which is excluded during isolation of II-III. This finding is extremely significant in view of the fact that competence factors, to date, have been shown to be synthesized by limited tissues: bovine pituitary and platelets.

As has been previously noted, quiescent cells are arrested in the G_{0} phase of the cycle. It was found, for example, that 3T3 cells became growth arrested 12 h prior to DNA synthesis. It was proposed that a critical and irreversible regulatory event occurred in this phase of the cycle (Scher, et al., 1979). Antoniades, et al. (1977), found that only a short exposure (4-6 h) to PDGF, a
platelet derived competence factor, was required to 'commit' the cells to division. Pledger, et al. (1977), went on to show that a second regulatory component, the progression factor, was necessary if PDGF-competent cells were to enter the S phase of the cell cycle. It is now clear that both factors are needed for an optimal proliferative response in mammalian cell cultures. It should be emphasized that FGF, which has been extensively characterized, only stimulates cells of mesodermal origin and that nonmesodermal cells do not require FGF for growth (Gospodarowicz and Moran, 1976). PDGF shows a similar spectrum of biological action (Scher, et al., 1980).

Paracrine regulation of organ growth clearly could be under the two-fold control of locally produced competence and progression factors. In addition, since competence activity is needed by only mesodermally derived cell populations, fibroblasts could produce large amounts of progression factor, inducing cell division in neighboring populations while not responding themselves due to low competence availability. Although only speculative at present, evidence exists to suggest that competence factors may play a role in differentiation. FGF has been shown to exert influence over the phenotypic expression of cells once they reach confluency. This is best demonstrated by its influence on the appearance of specific cell surface proteins such as fibronectin and CSP-60.
In order to explore the potential maturational influence of II-III, its effect on surfactant production was monitored. The radiolabeled choline incorporation experiments using mixed cell cultures (Figure 19) provide evidence against any maturational surfactant stimulating property of II-III. The lung is thought to contain approximately 40 different cell types in the adult. This fact necessitates a method of isolation of type-II cells prior to culturing (Hitchcock, 1980; Mason and Williams, 1980). The method of Keller and Ladda (1979) was modified as described in the Materials and Methods. This resulted in cultures which, on inspection by light microscopy, contained a majority of round, granular cells and which were 70% type-II cells as determined by transmission electron microscopy. In addition, choline incorporation into lipids was increased by glucocorticoids, an effect that has been a well-documented response of lung explants and mixed cell cultures.

In addition, the maturational influence of II-III was also monitored using the A549 cloned cell line. As previously noted the A549 cell line is derived from a human adenocarcinoma of the lung. Use of this bioassay system obviates the need to isolate type-II cells, as well as eliminating the interference of other cell types in culture. The morphological as well as biochemical
characteristics of the A549 cells have been extensively studied as a model for exploring the role of the type-II cell in lung development (Shapiro, et al., 1978). Nardone and Andrews (1979) reported that the A549 cell is a faithful model of pulmonary type-II cell as determined by the following criteria: 1) general ultrastructure as determined by transmission electron microscopy, 2) total phosphatidylcholine composition, 3) the pattern of incorporation of phosphatidylcholine precursors, 4) the production of phosphatidylcholine synthesized from radiolabeled choline is consistent with values reported for type-II cells. Mason and Williams (1980), however, disagree with Nardone and Andrews (1979) and conclude from their studies that the phospholipid composition of these tumor cells is significantly lower than for freshly isolated rat type-II cells.

In light of this, both culture systems were used in the present studies. Results for both the mixed cell and the A549 cultures showed no effect of II-II on choline incorporation into lipids. Thus, it would appear that the mitogens isolated via these procedures were distinct from the maturational factor described by Smith (1979, 1981).

The production by lung fibroblasts of a surfactant stimulating factor was further explored by chromatographing starting CSFM through Sephadex G-75. These experiments revealed the presence of a surfactant stimulating
substance at a $K_{av}$ value of 0.23 (Fig.22). This factor did not exhibit immunoactivity in the somatomedin-C radioimmunoassay, and differentially stimulated mitosis in mixed cell cultures. These data clearly demonstrate that the CEFA stimulating activity is distinct from the choline stimulating substance, which has a considerably higher molecular weight (>13000). Interestingly, the choline stimulating activity is also mitogenic in the mixed cell cultures but not in the CEFA bioassay. These data bear witness to a true paracrine factor which has both maturational and mitogenic properties and which is distinct from II-III. In addition, II-III does not appear to be mitogenic for type-II cells as attested to by the lack of mitogenic response in the mixed cell cultures.

SUMMARY AND CONCLUSIONS

Alescio and Piperno (1967) were the first to demonstrate that fetal lung epithelium required the presence of mesenchymal lung tissue. These investigators hypothesized the existence of an epithelial growth factor like the one demonstrated by Grobstein's (1953) studies on mouse salivary gland. In subsequent years the isolation of growth peptides has provided indirect support for Alescio's and Piperno's (1967) original hypothesis. The huge body of literature accumulating in the area of growth factors has begun to transform the classical concepts of the hormonal regulation of growth. It is now clear that, though blood
borne factors probably do play an important role in orchestrating organ growth, fine-tuned regulation probably occurs locally. Locally secreted peptides are considered to be paracrine factors if they exert their influence on neighboring populations of cells, and are considered autocrine factors if they secrete factors necessary for their own growth.

The results of these studies provide direct support for the paracrine model of lung development first conceived of by Alescio and Piperno in 1967. The lung-derived peptides which have been demonstrated in the current studies constitute several discrete components of the "inductive mesenchymal influence" postulated nearly 20 years ago by these investigators. These studies demonstrate the presence of a maturational/mitogenic factor, influencing type-II mixed cell cultures. In addition to this paracrine factor, II-III has been shown to play an autocrine role in stimulating proliferation of fetal lung fibroblasts, thereby increasing mesenchymal mass. Finally, these data suggest the existence of a locally produced competence factor which may be playing a critical role in regulating mitosis in the fibroblast population. The existence of this family of local fetal lung factors, synthesized and secreted in situ, provides the first step toward a true understanding of the mechanisms controlling embryonic development in general. In particular, these
studies begin to explain the surprising degree of autonomy previously described in lung organogenesis nearly 35 years ago.
BIBLIOGRAPHY


Enesco, M., and C.P. Leblond. (1962) Increase in cell number as a factor in the growth of the organs and tissues of the young male rat. J. Embryol. Exp. Mol. 10:530-538.


