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Factors Modifying DNA Synthesis by Lung Fibroblasts *in Vitro*[1] (41485)

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Abstract. DNA synthesis in guinea pig lung fibroblast cultures was shown to be stimulated by endotoxins, PGE_2, CTAP-III, CTAP-P_2, and insulin; indomethacin and cortisol partially reversed some of these effects. DNA synthesis in human lung fibroblasts was also markedly stimulated by CTAP-III, while the response to endotoxins was less striking.

Lung fibroblast cultures derived from the gas exchange component of pulmonary parenchyma provide a potentially useful *in vitro* model system for studying pulmonary connective tissue metabolism (1–4). This culture system permits one to study new synthesis of connective tissue by lung fibroblasts, and to study separately the replication of the cells and the chemical anatomy of the extracellular matrix materials secreted by pulmonary fibroblasts as these various activities may be modified by physiologic, pathologic, and pharmacologic agents.

In the present study, we focused on lung fibroblast proliferative responsiveness (measured by incorporation of [*methyl*-^{3}H]thymidine into fibroblast DNA) to relevant agonists of both exogenous and endogenous origin. Our interest in this aspect of pulmonary inflammation originates in the possibility that an expanding population of lung fibroblasts may be an important antecedent to accelerated fibrosis and deposition of extracellular matrix materials. Supporting this idea is evidence from experimental cotton pellet granulomas in rats showing a direct correlation between the amount of connective tissue DNA and the accumulating extracellular connective tissue matrix materials of the progressing inflammatory process (5). The inflammatory response in rat lung to intermittent oxygen exposure was also accompanied by elevated DNA values during the later "fibroblastic" phase of inflammation (6). Rabbit lung fibroblast cultures exposed to various dusts often responded with increased DNA synthesis in parallel with increased synthesis of extracellular matrix materials including collagen and glycosaminoglycans (7).

If fibroblast proliferation is important to mounting a destructive fibrotic process in pulmonary tissue, factors capable of accelerating or repressing lung fibroblast DNA synthesis may have practical significance. With this in mind, we have examined the effect of gram-negative endotoxins analogous to what might be released in lung during bacterial pneumonitis. Further, since immune complex-mediated injury to pulmonary endothelium results in local secretion of platelet-derived growth factors, we have examined the effect of two platelet-derived connective tissue activating peptides, CTAP-III (8, 9) and CTAP-P_2 (10). In addition, we examined the effects of prostaglandin E_2 (PGE_2), known to be secreted by lung fibroblasts (11).

Materials and Methods. *Culture methods.* Guinea pig lung fibroblasts were isolated by enzymatic disaggregation of lung parenchyma and plating at high density in plastic flasks as previously reported (1). Human lung fibroblast cultures were established in the same manner from normal tissue removed at lobectomy for neoplasm. Cultures derived in this way were propagated using medium F-12 (with 10% fetal calf serum, FCS 10%) or medium 1066, 90%: FCS 10%. Three different guinea pig lung fibroblast lines and one human lung fibroblast line were used in this study.

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Complete medium changes were carried out three times a week and trypsin dispersal was performed as required for propagation or study. Freezing procedures were as previously reported.

DNA synthesis was measured by determining the incorporation of [*methyl*-^{3}H]-thymidine into cells in microtiter well cultures utilizing 10^4 cells per culture (8).

Sources of mediators. CTAP-III was isolated from outdated human platelets by methods previously reported (8, 9). This cationic protein appears to be the major human platelet derived growth factor; it was essentially homogeneous in multiple PAGE systems and its sequence has been established (12). A minor platelet-derived growth factor, *CTAP-P*$_2$, is an anionic material which stimulates DNA and GAG synthesis in human synovial cells. *CTAP-P*$_2$ was partially purified as a by-product of the isolation of CTAP-III, i.e., from biologically active fractions which did not react with antisera to CTAP-III (10). The major contaminant identified in CTAP-P$_2$ preparations is albumin.

The enteric *endotoxins* were obtained from Difco Laboratory, Detroit, Michigan, while the *Klebsiella pneumoniae* LPS was a gift from Dr. A. I. Braude, University of California, San Diego. Crystalline bovine insulin was obtained from Sigma Chemical Company, St. Louis, Missouri, and polyinosinic:polycytidylic acid (Poly I:C) was a gift from Dr. A. A. Tytell, Ph.D., Merck Sharp and Dohme, West Point, Pennsylvania. Prostaglandin E_2 (PGE_2) was kindly provided by Dr. John E. Pike, The Upjohn Company, Kalamazoo, Michigan.

Results. *Endotoxin stimulation of DNA synthesis.* Bacterial endotoxin was shown to be capable of substantially stimulating the synthesis of DNA in lung fibroblast cultures (Table I). It is noteworthy that very small amounts of LPS were not uncommonly more stimulatory than higher concentrations. It is clear that among the potential inhibitors of DNA synthesis, that cycloheximide virtually ablated *basal synthesis,* indomethacin at clinically achievable concentrations was without effect, and cortisol exhibited marginal suppression of *basal* DNA synthesis.

It was noteworthy that both cortisol and indomethacin partially reversed the *stimulatory effect* of *E. coli* LPS on lung fibroblast DNA synthesis. The observations recorded for *E. coli*:026:B6 endotoxin was representative of the guinea pig lung fibroblast response to other endotoxins including: *S. typhosa*-0901, *E. coli*:0111:B4, *S. typhimurium,* and *S. minnesota.*

Of some interest was the observation (Tables I and II) that PGE_2, 1 μg/ml, signifi-

TABLE I. AGENTS MODIFYING ENDOTOXIN STIMULATION OF [^{3}H]DNA SYNTHESIS IN GUINEA PIG LUNG FIBROBLAST CULTURES[a]

Additives	[^{3}H]DNA (cpm/10^4 cells)[b]	Experimental/ control	*P*
0.15 *M* NaCl	3,787 ± 584	—	—
PBS[c]	4,271 ± 708	—	—
E. coli 026:B6, LPS, 1.0 μg/ml	24,380 ± 5935	6.4	<0.01
E. coli LPS, 50 μg/ml	14,045 ± 3684	3.7	<0.01
Cortisol, 1.0 μg/ml	2,781 ± 716	0.7	<0.05
Cycloheximide, 10 μg/ml	110 ± 17	0.03	<0.01
Indomethacin, 15 μg/ml	2,962 ± 1077	0.8	NS[d]
E. coli LPS, 50 μg + cortisol	4,799 ± 1980	1.3	<0.01
E. coli LPS, 50 μg + indomethacin	8,227 ± 1562	2.2	<0.02
E. coli LPS, 50 μg + cycloheximide	69 ± 21	0.02	<0.01
PGE_2, 1.0 μg/ml	7,032 ± 1859	1.9	<0.01

[a] Target cultures were GP-22 lung fibroblasts.
[b] Data are expressed as the mean ± 1 SD for four to six microcultures in this and subsequent tables.
[c] PBS refers to buffered saline containing 0.15 *M* NaCl plus 0.05 *M* phosphate buffer, pH 7.0.
[d] Not significant.

TABLE II. AGENTS MODIFYING PLATELET OR INSULIN STIMULATION OF [^{3}H]DNA SYNTHESIS IN GUINEA PIG LUNG FIBROBLAST CULTURES[a]

Additives	[^{3}H]DNA (cpm/10^4 cells)	Experimental/ control	P
0.15 M NaCl	7,826 ± 1718	—	—
PBS	6,921 ± 1711	—	—
CTAP-III, 21 μg/ml	39,123 ± 2006	5.0	<0.01
CTAP-III + indomethacin	23,183 ± 97	3.0	<0.01
CTAP-III + cortisol	35,722 ± 2418	4.6	NS[b]
Cortisol, 1.0 μg/ml	10,405 ± 868	1.3	<0.05
Indomethacin, 15 μg/ml	7,175 ± 412	0.9	NS
CTAP-P_2, 163 μg/ml	15,793 ± 1615	2.0	<0.01
CTAP-P_2 + indomethacin	8,967 ± 1792	1.1	<0.01
CTAP-P_2 + cortisol	10,821 ± 2375	1.4	<0.05
Insulin, 0.5 unit/ml	40,450 ± 3516	5.2	<0.01
Insulin, 5.0 unit/ml	42,258 ± 4311	5.4	<0.01
Insulin, 5 unit/ml + indomethacin	28,016 ± 973	3.6	<0.05
Insulin, 5 unit/ml + cortisol	47,519 ± 1364	6.1	NS
E. coli LPS, 026:B6, 50 μg/ml	11,263 ± 339	1.6	<0.02
PGE_2, 1.0 μg/ml	17,705 ± 1376	2.3	<0.01
PGE_2, 5 μg/ml	11,545 ± 1366	1.5	<0.01

[a] The GP-20 lung fibroblast line served as target cultures in this experiment.
[b] Not significant.

cantly stimulated DNA synthesis. Not all experiments demonstrated PGE_2 stimulation of DNA synthesis; on occasion, very high concentrations (5 μg/ml) were inhibitory while 0.1 μg/ml was stimulatory.

Stimulation of DNA synthesis by platelet factors and insulin. It was clear that both platelet factors, CTAP-III and CTAP-P_2, stimulated DNA synthesis in guinea pig lung fibroblast cultures to an extent comparable to that in the endotoxin experiments (Table II). In the present experiment, the specific stimulation by CTAP-III seemed to be greater than that generated by CTAP-P_2, although the less purified state of the CTAP-P_2 makes such calculations imprecise. Indomethacin significantly reduced the stimulatory effect of CTAP-III while cortisol was apparently ineffective. Both indomethacin and cortisol significantly reversed the stimulatory effect of the minor platelet factor, CTAP-P_2.

Bovine insulin in pharmacologic concentrations was shown to be a potent stimulator of DNA synthesis in these cell cultures, an effect which was resistant to cortisol suppression. On the other hand, indomethacin was able to partially suppress the insulin-stimulated increase in DNA synthesis.

Stimulation of DNA synthesis in human lung fibroblast cultures. Lung fibroblast cultures of human origin showed a marked increase in DNA synthesis on exposure to the major platelet mitogen CTAP-III, and relatively minor levels of stimulation by the synthetic polynucleotide, Poly I:C, (Table III).

Insulin caused a minor increase in DNA synthesis, cycloheximide ablated DNA synthesis, and cortisol exhibited a borderline stimulatory effect. The enteric endotoxins had no significant effect on DNA synthesis; *K. pneumoniae* LPS caused a significant, but minor, stimulation of lung fibroblast DNA synthesis. PGE_2 (data not shown) did not stimulate DNA synthesis in the human lung fibroblast cultures.

Discussion. In general, endotoxin stimulation of DNA synthesis was greater in guinea pig lung fibroblast cultures than in our human line. Earlier studies demonstrated that the lipid A core of endotoxins was a potent stimulator of glycosaminoglycan synthesis in human synovial cultures (13), and recently lung fibroblasts were

TABLE III. STIMULATION OF [^{3}H]DNA SYNTHESIS BY HUMAN LUNG FIBROBLASTS

Mediator or vehicle	[^{3}H]DNA (cpm/10^4 cells)	Experimental/ control	*P*
Experiment 1			
0.15 *M* NaCl	1,074 ± 105	—	—
CTAP-III, 25 μg/ml	11,069 ± 4532	10.3	<0.01
Poly I:C, 50 μg/ml	2,243 ± 414	2.1	<0.01
Experiment 2			
0.15 *M* NaCl	11,417 ± 1060	—	—
CTAP-III, 19 μg/ml	35,636 ± 2076	3.12	<0.01
Insulin, 0.4 μg/ml[a]	16,669 ± 1634	1.46	<0.01
Cortisol, 1.0 μg/ml	14,181 ± 1197	1.24	<0.05
Cycloheximide, 10 μg/ml	1,944 ± 273	0.17	<0.01
E. coli 026:B6 endotoxin 0.5 μg/ml	12,962 ± 1736	1.14	NS
S. tymphimurium endotoxin, 5.0 μg/ml	12,943 ± 1579	1.22	NS
Klebsiella endotoxin, 0.5 μg/ml	14,240 ± 733	1.25	<0.01

[a] 0.4 μg corresponds to 10 munit. Normal human insulin values range from 4 to 10 μunit/ml of serum.

shown to exhibit increased GAG synthesis on exposure to endotoxins (14). Whether this relative resistance of our human lung fibroblast line to endotoxin stimulation of DNA synthesis is a peculiarity of the cell line or representative of such cells remains to be established.

These data demonstrate that CTAP-III, a growth factor derived from human platelets, markedly stimulates DNA synthesis in lung fibroblast cultures of guinea pig and human origin. Since CTAP-III is secreted by platelets in the course of the aggregation-granule release process (8, 15), it is likely that relatively large concentrations of this mediator flood local areas of injury. While it is possible that CTAP-P_2 is also presented to injured tissue in the same manner, there is yet no unambiguous data on its secretion mechanism. One may speculate that an injurious process in lung, be it physical, chemical, immunologic, or microbiologic, may initiate fibroblastic proliferation in part through this mechanism. In guinea pig lung cultures, it was possible to partly suppress this response with the anti-inflammatory agent indomethacin in "clinically relevant" concentrations.

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