Fibroblast Prostaglandin E_2 Synthesis

PERSISTENCE OF AN ABNORMAL PHENOTYPE AFTER SHORT-TERM EXPOSURE TO MONONUCLEAR CELL PRODUCTS

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ABSTRACT Acquired abnormalities of connective tissue metabolism in inflammatory diseases often persist when lesional tissue is maintained in in vitro culture. Although connective tissue cells are exposed to inflammatory cell-derived mediators in vivo and such mediators have been shown to alter connective tissue cell behavior, it is unclear whether the persistence of metabolic defects in vitro could result from remote in vivo exposure to these mediators. An in vitro model was used to test whether transient exposure of normal fibroblasts to inflammatory mediators could lead to metabolic alterations that persist during in vitro culture. Short-term exposure of human foreskin fibroblasts in vitro to supernates of mitogen-activated peripheral blood mononuclear cells led to persistent abnormalities of prostaglandin E_2 (PGE_2) metabolism. Fibroblasts previously exposed to mononuclear cell products synthesized more than twice as much PGE_2 when stimulated compared with similarly stimulated but previously unexposed control fibroblasts of the same strain. The enhanced PGE_2 synthesis persisted for as long as 20 wk and 19 cell generations after the original exposure to mononuclear cell products. Exposure of fibroblast populations to mononuclear cell products may, thus, lead to metabolite alterations that are still evident after multiple cell generations.

INTRODUCTION

The constellation of metabolic events characterizing connective tissue cell behavior in the synovium of rheumatoid arthritis has been called "connective tissue activation" (1, 2). The phenotypic expression of this activation includes general activation of metabolic processes manifested by increased glucose uptake and lactic acid formation (1–3) as well as specifically enhanced synthesis of prostaglandins (PG)[1], collagenase, and collagen (4, 5). Cell cultures derived from rheumatoid synovial tissue display the "activated" phenotype described above (2). Furthermore, the abnormal metabolic behavior of these cells persists for long periods and multiple cell generations in culture. Thus, increased synthesis of collagenase, abnormal growth kinetics, hyporesponsiveness to cortisol, and hyperresponsiveness to stimulation of prostaglandin E_2 (PGE_2) synthesis persist for months in culture (1–5).

Recent studies support the concept that products of lymphocytes and monocytes play a role in connective tissue cell activation. Lymphocyte products stimulate fibroblast proliferation and collagen synthesis (6). Monocyte products stimulate fibroblast and/or synovial cell PG and collagenase production (7–9) and suppress fibroblast proliferation, the latter resulting from stimulation of endogeneous PGE_2 synthesis (9). The PG stimulatory activity is released by unstimulated monocytes but increased activity is released with mitogen stimulation in the presence of lymphocytes (9, 10). The stimulation of fibroblast PGE_2 synthesis is mediated by a 12–20,000-mol wt peptide product of human monocytes that is probably identical with interleukin 1 (IL-1) or lymphocyte-activating factor (11). One would not expect a priori, however, that exposure of connective tissue cells to such mononuclear

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; MC, mononuclear cell; PG, prostaglandin(s); PHA, phytohemagglutinin; SN, supernate.

cell (MC) mediators in vivo would lead to a metabolic abnormality that not only persists in vitro but is heritable for multiple cell generations.

In the present studies the possibility was investigated that short-term exposure of normal fibroblasts to MC products in vitro might lead to persistent abnormalities of PGE_2 metabolism. When fibroblasts were so exposed, there resulted populations that demonstrated abnormally high stimulated PGE_2 synthesis (compared with control unexposed cells of the same strain). Furthermore, this phenotype persisted for as long as 20 wk and 19 cell generations after the original exposure to MC products.

METHODS

Fibroblast cultures were established from collagenase digests of neonatal foreskin obtained at routine circumcision (11). Cultures maintained in humidified 5% CO_2/95% air were fed twice weekly with basal Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 25 mM HEPES buffer, and penicillin-streptomycin-amphotericin B (100 U, 100 μg, and 0.25 μg/ml, respectively) (complete Eagle's medium). Fibroblasts were split (2:1) at confluence, usually every 7–10 d.

Supernate (SN) preparation. SN of phytohemagglutinin (PHA)-stimulated human peripheral blood MC were prepared as previously described (9). Briefly, MC were isolated by Ficoll-diatrizoate density gradient centrifugation of heparinized blood from healthy volunteers. MC were cultured in 17 × 100-mm polypropylene tubes at 10^6 cells/ml in RPMI 1640 (supplemented with FCS, glutamine, HEPES buffer, and antibiotics-antimycotic as described above) containing 2.5 μg/ml of PHA. 3-d culture SN were harvested by centrifugation, passed through a 0.20 μm filter and stored at −20°C until use.

Transient exposure of fibroblasts to MCSN. For each strain tested, fibroblasts from several culture dishes of a single passage (4–6th) were harvested by exposure to 0.25% trypsin-0.2% EDTA, pooled, and seeded into replicate 60-mm culture dishes containing either RPMI 1640/10% FCS (control) or the same medium containing MCSN (SN-exposed). Control and SN-exposed fibroblasts were maintained in parallel (fed twice weekly and split at confluence) except that at each feeding SN-exposed fibroblasts received fresh culture medium containing MCSN; control fibroblasts received medium alone.

Assay culture. Following the periods of SN-exposure (2–12 wk for different strains), control and SN-exposed (experimental) fibroblasts of each strain were returned to complete basal Eagle's medium and maintained in culture as described above. At intervals after return to normal culture, control and experimental fibroblasts of each strain were assayed for base-line and stimulated PGE_2 synthesis as previously described (9). Confluent dishes of control/experimental fibroblasts were synchronized by overnight incubation in serum-free medium, harvested by trypsin-EDTA exposure, washed, and resuspended in complete RPMI. Cells were then seeded in 100 μl volumes into replicate wells (5 × 10^3 cells/well) of 96-well culture plates; 100 μl volumes of complete RPMI (base-line PGE_2 synthesis), 1.5% MCSN, or 25% MCSN (vol/vol in complete RPMI) (stimulated PGE_2 synthesis) were added to quadruplicate wells. Following 48 h of culture, 100 μl of medium was aspirated from each well; medium from the first and second and third and fourth wells of each set were pooled to yield duplicate 200-μl samples. Samples were frozen at −20°C until assay.

Parallel cultures of each control/experimental pair were assayed for incorporation of [^{3}H]thymidine as an index of fibroblast proliferation (9). Triplicate or quadruplicate wells identical to those established for PGE_2 synthesis were pulsed after 42 h of culture with [^{3}H]thymidine (0.5 μCi/well). Cells were harvested after 48 h on glass fiber filters using a semi-automated cell harvester (Otto Hiller Co., Madison, WI) and washed with distilled water; filter discs were immersed in Econosol and counted in a liquid scintillation counter (Packard Tri-Carb, Packard Instrument Co., Downers Grove, IL). We have previously shown that under these conditions [^{3}H]thymidine incorporation correlates well with direct cell counts (9).

Radioimmunoassay (RIA) for PGE. RIA for PGE_2 was performed as previously described (12) using a commercially obtained antibody to PGE_2 (Seragen Inc., Boston, MA). This antibody cross-reacts completely with PGE_1, 10.6% with PGA_2, 2.7% with $PGF_{2\alpha}$, and 0.3% with 6 keto-$PGF_{1\alpha}$. Under the culture conditions used, PGE_2 accounts for >80% of [^{14}C]arachidonic acid incorporation into PG as determined by thin-layer chromatography of (unstimulated or stimulated) culture SN (unpublished observations). Each one of duplicate samples was assayed at two or more dilutions; standard deviations of assays at different dilutions and of duplicate samples were within 10% of the mean.

Reagents. Chemicals and supplies were obtained from the following sources: culture media and supplements, Gibco Laboratories, Grand Island Biological Co. Grand Island, NY; FCS, KC Biologicals, Lenexa, KS; culture dishes and plates, Costar, Bellco Glass Co, Vineland, NJ; PHA, Burroughs Wellcome, Research Triangle Park, NC; trypsin, EDTA, buffer, Sigma Chemical Co, St Louis, MO; [^{3}H]thymidine, [^{3}H] PGE_2, and Econosol, New England Nuclear, Boston, MA; PG standards were a kind gift of Mr. P. Coughlin, Upjohn Co. Kalamazoo, MI.

RESULTS

Fibroblasts from six human foreskin strains were grown for 2 wk in either normal culture medium (control) or the same medium containing 6% (vol/vol) crude SN of PHA-stimulated peripheral blood MC (SN-exposed, experimental). During this period, fibroblasts grown in the presence of MCSN displayed slower growth and were subcultured only once (2:1 split); control fibroblasts were subcultured twice. Paired confluent control/experimental culture dishes were incubated overnight in serum-free medium and PGE_2 synthesis in the control and experimental fibroblasts was assessed.

Base-line (unstimulated) PGE_2 synthesis was similar in control fibroblasts and fibroblasts that had been previously SN-exposed (1.3±0.3 vs. 1.8±0.6 ng PGE_2/ml, respectively, mean±SEM, $n = 6$, $P > 0.05$) (Fig. 1). The low level of base-line PGE_2 synthesis by previously SN-exposed fibroblasts demonstrates that the overnight culture in serum-free medium allowed recovery from SN-mediated stimulation of PGE_2 synthesis during the previous culture. Stimulated PGE_2 synthesis was as-

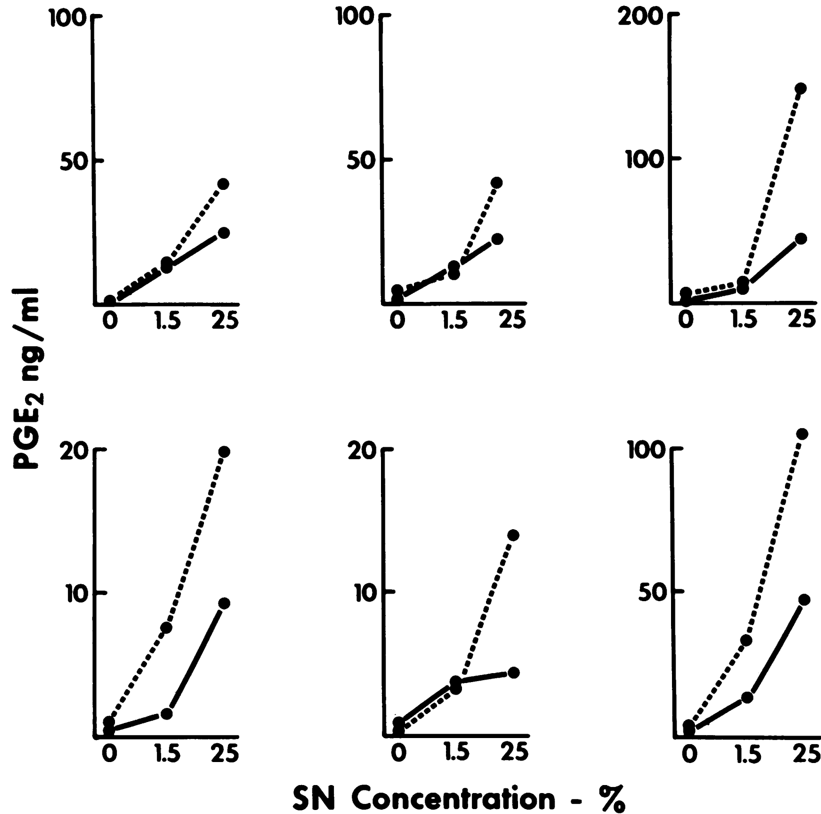

FIGURE 1 Comparison of PGE_2 synthesis by fibroblasts grown for 2 wk in normal media vs. those grown in MCSN. Control/experimental fibroblasts were synchronized by overnight incubation in serum-free medium and seeded into replicate microwells of 96-well culture dishes (5×10^3 cells/well). PGE_2 synthesis was assessed in 48-h cultures of unstimulated (0% SN) and stimulated (1.5 and 25% SN) fibroblasts. Each graph shows PGE_2 concentrations in the culture medium of control (solid line) and experimental fibroblasts from the same strain (dotted-line).

sessed by adding MCSN to the fibroblast cultures (9). With 1.5% MCSN, PGE_2 synthesis increased in both control and experimental fibroblasts; experimental fibroblasts from two of six strains tested synthesized greater amounts of PGE_2 than the paired control culture. In the presence of 25% MCSN, PGE_2 synthesis by previously SN-exposed (experimental) fibroblasts exceeded that of control cells for each of the six strains tested (mean control 26.8±6.6 ng PGE_2/ml vs. mean experimental 62.6±20.0 ng/ml, $n = 6$, $P < 0.01$). Thus, experimental (previously SN-exposed fibroblasts) were hyperresponsive in terms of PGE_2 synthesis to subsequent challenge with fresh MCSN.

Stimulation of PGE_2 synthesis occurred despite suppression of cellular proliferation (see below and reference 9) and was not, therefore, due merely to an increase in cell number. PGE_2 in the culture medium was synthesized by the fibroblasts and not due to passive carry-over of PGE_2 in the MCSN; when fibroblasts were treated with 25% MCSN in the presence of 1 μg/ml indomethacin, PGE_2 concentrations in the culture medium were <1 ng/ml (data not shown). Cells exposed for 2 wk to PHA alone displayed base-line and stimulated PGE_2 synthesis similar to control cells indicating that the difference between control and experimental fibroblasts was due to prior exposure to MC-derived factors in the SN and not to the content of PHA in the SN (data not shown).

Separate experiments were performed to determine whether the enhanced stimulated PGE_2 phenotype of experimental cells was a temporary consequence of recent SN-exposure or whether it reflected a constituent change in the responding fibroblast population. Three fibroblast strains were transiently exposed to MCSN and then maintained in normal culture media. At regular intervals fibroblasts were sampled for base-line and stimulated PGE_2 synthesis. Control cells of each strain were maintained and sampled in an identical manner.

Previously SN-exposed (experimental) fibroblasts,

even after prolonged culture in normal media, consistently demonstrated higher stimulated PGE_2 synthesis than control cells (Table I). Enhanced stimulated PGE_2 synthesis by experimental cells persisted for up to 20 wk and 19 cell generations in culture and was greater than matched control cells at 16 of 17 time points tested for the three cell strains ($P < 0.001$, Wilcoxin test for paired samples). Over the interval tested, stimulated PGE_2 synthesis by experimental fibroblasts was 314±111, 220±57, and 199±33% (mean±SEM) of control fibroblasts for strains R, OW, and T respectively.

We have previously shown that SN of unstimulated MC stimulate PGE_2 synthesis of normal foreskin fibroblasts although to a lesser extent than SN of PHA-stimulated MC (9). Since experimental fibroblasts had been previously exposed only to PHA-stimulated MCSN, we examined whether the heightened PGE_2 synthetic response of these cells was restricted to stimulated vs. unstimulated MCSN. Fibroblasts from three experimental strains synthesized more PGE_2 compared with matched control strains whether stimulated or unstimulated SN was used (Table II). For both experimental and control strains, PGE_2 synthetic response to unstimulated SN was less than to PHA-stimulated SN. The difference in potency between stimulated and unstimulated-SN was not due to passive carry-over of PHA in the stimulated-SN. As demonstrated previously (9), PHA alone (at the concentration passively carried over in the stimulated-SN) had little effect on PGE_2 synthesis.

Absolute levels of stimulated PGE_2 synthesis in both control and experimental substrains showed considerable variability. Such variability may be due in part to differences in potency of MCSN preparations used at different time points with different strains although, in our experience, a single fibroblast strain assayed on different days with the same MCSN preparation shows considerable variability (unpublished observations). In each case, matched control/experimental substrains at a given time point were tested together using a single MCSN preparation. No consistent effect of cell passage number was observed on absolute PGE_2 synthesis or on the difference between control and experimental members of a pair. When data for weeks (1 + 2), (4 + 8), and (16 + 20) were analyzed separately, stimulated PGE_2 synthesis by experimental fibroblasts was, respectively, 183±34% ($P = 0.06$), 211±43% ($P < 0.05$), and 217±56% ($P < 0.025$) that of control fibroblasts.

Cellular proliferation. Control and experimental fibroblasts from each strain were compared for cellular proliferation measured by [^{3}H]thymidine incorporation. Base-line [^{3}H]thymidine incorporation (i.e., in the absence of MCSN) was greater for experimental (previously MCSN-exposed) fibroblasts than control fibro-

TABLE I
Persistence of Abnormally High Stimulated PGE_2 Synthesis in Fibroblasts Exposed Briefly to MC Products

	Duration of normal culture*						
	Weeks						
	0	1	2	4	8	16	20
	ng PGE_2/ml						
Strain R							
Control	15.0	150.0	72.5	9.8	34.0	27.4	9.1
Experimental	150.0	250.0	160.0	38.5	50.0	37.6	11.9
Strain OW							
Control	4.3	6.7	ND	9.0	13.6	15.5	3.8
Experimental	9.9	5.7	ND	14.75	17.0	31.9	19.5
Strain T							
Control	ND	5.0	17.8	ND	12.0	11.2	51.3
Experimental	ND	15.4	24.4	ND	27.5	24.0	53.2

* Cells from each of three foreskin fibroblast strains were put into normal culture medium (control) or medium containing 6% MCSN (experimental) for 12 wk (strain R) or 2 wk (strains OW, T). Control and experimental fibroblasts of each strain were then returned to normal culture medium and assayed at intervals for PGE_2 synthesis when stimulated by 25% MCSN. Control and experimental substrains were treated identically from 0–20 wk and underwent 14, 15, and 19 subpassages (strains R, OW, and T, respectively) during this period. ND, not determined.

TABLE II
PHA-stimulated and Unstimulated MCSN Preferentially Enhance PGE_2 Synthesis by Experimental Fibroblasts

Addition to culture	Fibroblast population*		
	Control	Experimental	
	PGE_2 ng/ml culture medium		
PHA (0.62 μg/ml)	1.0±0.1‡	1.1±0.2	$P < 0.01$
Unstimulated MCSN (25% vol/vol)	4.9±0.6	8.1±1.9	$P < 0.01$
Stimulated MCSN (25% vol/vol)	8.7±1.3	13.2±3.1	

* Fibroblasts were cultured overnight in serum-free medium, then cultured for 48 h in MCSN or PHA-containing medium. Fibroblasts cultured in RPMI alone yielded PGE_2 concentrations of 0.4±0.1 ng ml for both control and experimental fibroblasts.
‡ Each value represents the mean (±SEM) of duplicate determinations for three separate control/experimental strain pairs.

blasts in all three strains OW, R, and T. MCSN suppressed proliferation of both experimental and control substrains but proliferation in the presence of MCSN was greater in experimental fibroblasts from strains OW and T (Table III). The differences in [^{3}H]thymidine incorporation between experimental and control fibroblasts were statistically significant when data from all three strains were combined. The increased stimulated PGE_2 synthesis of experimental cells did not seem, however, to be related to enhanced proliferation relative to control cells. When the ratio of experimental/control [^{3}H]thymidine incorporation was plotted against experimental/control PGE_2 synthesis no significant correlation was observed (n = 17; r = 0.03; P = 0.9).

TABLE III
*Proliferation of Experimental vs. Control Fibroblasts**

Strain	n	[^{3}H]Thymidine incorporation of experimental fibroblasts			
		Without added MCSN		With 25% MCSN	
			P‡		P‡
OW	7	119±13	NS	139±23	NS
R	7	138±30	NS	101±9	NS
T	7	143±9	NS	149±28	NS
All	21	133±13	<0.025	130±13	<0.05

* Each strain was assayed at 0, 1, 2, 4, 8, 16, and 20 wk after return to normal culture medium. [^{3}H]Thymidine incorporation for experimental fibroblasts at each time point was normalized to percentage of matched control culture. Results are expressed as mean percentage of control±SEM for seven time points for each strain. [^{3}H]Thymidine incorporation ranged from 5,000 to 22,000 cpm for control cells.
‡ Wilcoxon test for paired samples for experimental vs. control [^{3}H]thymidine incorporation where control = 100%. NS = $P > 0.05$.

We have previously shown that a 12–20,000-mol wt product of monocytes, present in supernates of PHA-stimulated MC cultures, suppresses fibroblast proliferation by stimulation of endogenous PGE_2 synthesis (9). We tested whether fibroblasts that had proliferated in the presence of a 2-wk MCSN exposure might subsequently be less susceptible to growth suppression on reexposure to MCSN. Control and experimental fibroblasts from six strains (OW, T, and four of the strains in Fig. 1) were assayed for base-line and MCSN-modulated [^{3}H]thymidine incorporation immediately after 2 wk of SN-exposure (i.e., at "0" wk but after overnight incubation in serum-free medium). MCSN (25% final concentration) suppressed [^{3}H]thymidine incorporation of both control and experimental fibroblasts; proliferation of experimental fibroblasts was suppressed to a lesser extent (25±8.8% vs. 42±7.6%, mean±SEM, $P < 0.05$). The difference in MCSN-mediated suppression of control vs. experimental fibroblast proliferation did not persist, however, in experimental cells of strains OW and T after they had been maintained in normal culture medium (n = 12, 36±7.0 and 36±6.2% suppression for control and experimental fibroblasts, respectively from strains OW and T assayed at 1, 2, 4, 8, 16, and 20 wk of normal culture, i.e., weeks after SN-exposure).

DISCUSSION

The results of these studies demonstrate that fibroblast populations exposed transiently to MC-derived products subsequently show an enhanced PGE_2 synthetic response to MCSN. This effect is present not only immediately after the transient exposure (Fig. 1) but persists after previously exposed fibroblasts are maintained in prolonged normal culture. The immediate hyperresponsiveness of experimental fibroblasts dem-

onstrated in Fig. 1 is the reverse of what might be expected. Dayer et al. (13) have demonstrated that the PGE_2 synthetic response of synovial fibroblasts exposed to MC-conditioned media is attenuated when the same cells are rechallenged with MCSN. Such "down-regulation" was likely avoided by overnight incubation of cells in serum-free medium before assay.

Products released by monocytes and lymphocytes have previously been shown to modulate a variety of connective metabolic functions. We have demonstrated for the first time that exposure of fibroblasts to MC products not only influences metabolism acutely but may also lead to metabolic alterations in the fibroblast that persist after removal of the MC-derived products. Further, the metabolic alterations are evident in the in vitro progeny of the exposed fibroblast populations.

Several mechanisms might be postulated to explain the emergence of fibroblast populations with a persistent and hereditable (in vitro) phenotype of enhanced SN-stimulated PGE_2 synthesis after transient exposure to MC mediators. Populations of fibroblasts as well as other cell types are known to be metabolically heterogeneous (14, 15). For example, clones of rabbit synovial fibroblasts isolated from a single synovial spectrum vary in both PGE_2 and collagenase synthetic capacity (14) and clones of fibroblasts isolated from cultures of human dermis show marked heterogeneity in collagen biosynthetic capacity (15). Indeed, we have shown that fibroblast clones derived from a single foreskin demonstrate marked heterogeneity in both proliferation and the PGE_2 synthetic response to MCSN.[2] Furthermore the clonal phenotypes are maintained as the clones are passaged in culture. It is possible, therefore, that the high stimulated PGE_2 synthesis of experimental fibroblasts noted above resulted from selective overgrown of high PGE_2-producing populations during the period of SN exposure. If such were the case, one might expect that the experimental (SN-exposed) fibroblasts would show accelerated growth relative to control fibroblasts in the presence of MCSN. In fact this difference was seen, but only when experimental fibroblasts were compared with matched controls immediately after the period of SN exposure.

High PGE_2 producing subpopulations of fibroblasts might, thus, arise as a consequence of immunologically mediated clonal selection, resulting from altered sensitivity to PGE_2 itself or to one or more growth suppressive/growth stimulatory mediators released by MC. Ko et al. (16) indeed found that human gingival fibroblasts are heterogeneous with regard to sensitivity to growth suppressive effects of PGE_2 and that exogenous PGE_2 could lead to disappearance of the PGE_2-sensitive population. If immunologically mediated selection is occurring, it is not immediately apparent why it should select for a high PGE_2-producing phenotype in each strain tested. One possible explanation is that those fibroblast subpopulations with the greatest PGE_2 synthetic response to MC-derived mediators might also be the most resistant to PGE_2-mediated antiproliferative effects.

An alternative mechanism for persistent abnormal phenotype after short-term SN exposure may be direct or indirect effects of MC products on gene content or expression. For example, MC products might lead to activation of latent virus in the fibroblast populations. However, fibroblasts that were transiently (2–12 wk) exposed to MC products did not undergo morphological changes and did not show growth characteristics different from matched control populations of the same strain. Furthermore, experimental fibroblasts, like their control pair, showed normal senescence in culture with cessation of in vitro proliferation after $\sim$30 cell generations.

It is also possible that some factor in MCSN acquired during the period of short-term exposure was carried through along multiple-cell generations. Buckingham and Castor (17) previously demonstrated that normal synovial fibroblasts exposed to extracts of gram-negative bacteria acquired "rheumatoid behavior" that persisted for almost 6 wk in culture. The altered rheumatoid phenotypes included enhanced glucose uptake and lactate output as well as increased synthesis of hyaluronic acid and persisted in exposed normal human synovial fibroblasts for several cell generations in culture. The authors hypothesized that the persistent metabolic abnormality resulted from carriage of bacterial endotoxin with each subculture. It is unlikely that endotoxin introduced by MCSN was responsible for the differences between control and experimental fibroblasts in the present report. The levels of endotoxin present in the MCSN used were the same as those present in our FCS-containing culture media (10^{-4} to 10^{-5} mg/ml by limulus amoebas lysate assay kindly performed by Dr. Frederick Rickles, University of Connecticut School of Medicine). Culture media of paired control and experimental fibroblasts contain identical amounts of endotoxin.

Our results suggest that mediators released by MC not only directly affect connective tissue metabolism but may lead to permanent alterations of connective tissue cell populations. Such permanent alterations could arise via clonal selection of fibroblast subpopulations. These mechanisms may be important not only in the genesis of "activated" synovial cells in inflammatory arthritides but also in such disorders as scleroderma, where fibroblasts from lesional tissue have been

[2] Korn, J. H. Submitted for publication.

shown to persistently synthesize abnormally high levels of collagen (18). The studies reported here provide an in vitro model that may be useful in exploring the role of immune cell-connective tissue cell interactions and the role that the interactions play in perpetuating the connective tissue lesion in inflammatory diseases.

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