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Prostaglandin E_2 elicits a morphological change in cultured orbital fibroblasts from patients with Graves ophthalmopathy

(connective tissue/Graves disease/inflammation/impedance/electrode)

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ABSTRACT **Fibroblasts derived from distinct anatomical regions appear to differ in regard to their behavior in culture. These differences may reflect functions of these cells *in vivo* that are tissue specific. Moreover, intrinsic differences in fibroblasts may underlie the site-specific connective tissue manifestations associated with systemic disease. We have demonstrated previously that orbital fibroblasts exhibit different cytokine response domains and protein synthetic programs when compared to those emanating from the skin. In the present communication, we demonstrate that prostaglandin E_2 (PGE_2) elicits in cultured human orbital fibroblasts from patients with Graves ophthalmopathy a rapid and dramatic change in cell morphology *in vitro* as assessed by phase-contrast and scanning electron microscopy. The central areas of the cells become elevated with respect to the plane of the substratum and are stellate, with long processes that touch neighboring cells. These changes occur within 6 hr of prostanoid addition to culture medium at an apparent concentration threshold of ≈10 nM. Shape changes are accompanied by marked alterations in monolayer impedance as assessed by electric cell–substrate impedance sensing as described previously. Both morphologic and impedance changes elicited by PGE_2 revert over 24 hr toward those found in untreated cells despite the continued presence of the prostanoid in the culture medium. In contrast, dermal fibroblasts fail to respond to PGE_2. These observations define a previously unrecognized phenotypic attribute of orbital fibroblasts. Intrinsic differences in these cells may account for the anatomic site-selective vulnerability of the orbit in Graves ophthalmopathy. The culture system described here may be useful for studying the morphogenic actions of prostanoids.**

Fibroblasts from different anatomic regions of the human body exhibit characteristic attributes when maintained in primary cell culture. In particular, the ability of a wide range of compounds to elicit biological responses appears to vary among fibroblast populations (1–4). These differences observed in culture suggest the possibility that fibroblasts perform tissue-specific functions *in vivo*. Cells derived from the orbital connective tissue display responses to several molecular factors that set them apart from dermal fibroblasts (2–4). For instance, thyroid and glucocorticoid hormones regulate the rate of hyaluronan synthesis in dermal fibroblasts (5, 6); however, neither class of hormone alters the production of this macromolecule in orbital fibroblasts (7). In contrast, interferon γ upregulates glycosaminoglycan accumulation in orbital but not in dermal cultures (8). Thus, there is reason to suspect that the metabolism of orbital fibroblasts might be differentially regulated by these and perhaps other factors *in situ*.

In this paper, we demonstrate an ability of prostaglandin E_2 (PGE_2) to elicit a rapid and pronounced change in the morphology of orbital fibroblasts from individuals with Graves ophthalmopathy. The alteration, from a characteristic fibroblast-like shape to a stellate appearance, could be visualized by both light and scanning electron microscopy. These changes are accompanied by a dramatic decrease in impedance of the cultured orbital fibroblast monolayers when measured with electric cell–substrate impedance sensing (ECIS). Dermal fibroblasts fail to exhibit shape changes in response to PGE_2. These results suggest another major difference in the phenotypic makeup of orbital fibroblasts compared to those originating from the integument.

MATERIALS AND METHODS

Materials. Eagle's medium, fetal bovine serum (FBS), and other cell culture reagents were obtained from GIBCO. PGE_2 and 8-BrcAMP were supplied by Sigma. All other reagents used were of the highest purity commercially available.

Cell Culture. Orbital and dermal fibroblasts were obtained from individuals without or with Graves disease. Institutional review board approval at the Albany Medical College was obtained to conduct these studies. The tissue explants were placed in plastic culture dishes and covered with Eagle's medium supplemented with Earle's salts, FBS (10%), glutamine, and antibiotics as described (9). Cultures were maintained in a 37°C humidified incubator with 5% CO_2/95% air. Fibroblast monolayers were passaged serially by gentle disruption with trypsin/EDTA, and some culture material was stored long term in liquid N_2. The doubling time for these cells was ≈1 day. For experiments, cells were <13 passages from culture initiation. The medium was changed every 3–4 days.

Phase-Contrast Microscopy. Fibroblasts were inoculated on sterilized glass coverslips (22 × 22 mm) at a density of 10^4 cells per coverslip and were allowed to attach for at least 1 day prior to any experimental manipulations. Cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS; pH 8.0). The coverslips were washed twice with PBS and mounted on glass slides with glycerol/PBS (1:1). Microscopy was performed using a Nikon Labophot equipped with a 35-mm camera. Photographs were taken on Kodak Tri-Max 400 film. The final magnification was ×400.

Scanning Electron Microscopy. Cell cultures were allowed to proliferate on glass coverslips, treated, and then fixed with

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Abbreviations: ECIS, electric cell–substrate impedance sensing; PGE_2, prostaglandin E_2.

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4% formaldehyde and 1% glutaraldehyde in a phosphate buffer. After secondary fixation in 2% osmium tetroxide, cells were dehydrated in ethanol and hexamethyldisilazane, coated with gold, and examined with a JEOL 6100 scanning electron microscope (10).

Impedance Measurement by ECIS. The measurement of fibroblast morphology when maintained as a monolayer was based on the technique developed and described previously (11–16). Details concerning the instrumentation have appeared recently (17). Briefly, in this system, cells are adherent to gold electrodes and culture medium serves as an electrolyte. An approximately constant current source applies an ac signal of 1 μA between a small active electrode and a larger counter electrode, while the voltage is monitored with a lock-in amplifier. Voltage and phase data are stored and processed with a personal computer. The same computer controls the output of the amplifier and switches the measurement to different electrodes in the course of an experiment.

When cells attach and spread on the gold electrodes, the insulating plasma membranes block the current path, and the impedance increases severalfold, depending on the cell type. To measure this change in impedance it is important that the active electrode is small in area (10^{-3} cm^2); otherwise, the solution resistance will dominate the measurement and mask the effect caused by the cells on the electrodes. The impedance will continue to fluctuate after the cells reach confluence, because of small changes in cell morphology.

Electrode Preparation. ECIS electrode arrays consisted of a row of five tissue culture wells mounted on a slide (1 × 3 in). The following fabrication steps are used: a 50-nm gold layer is first sputtered on 20-mil polycarbonate films. The gold layer is patterned by standard photolithography procedures to form contact pads, a large counter electrode, and an area that will finally include the active ECIS electrode. A 6-μm-thick layer of photoresist is spread over the patterned gold surface via spin coating. Again, using standard photolithographic procedures, the photoresist is selectively removed from areas of the slide including the 250-μm-diameter active electrodes. The photoresist is hard baked and finally cleaned and sterilized using an oxygen plasma etching procedure. To complete the arrays, sterile glass cloning cylinders (10 × 10 mm) are mounted to the plastic slide using a tissue culture-compatible silicone adhesive. The completed array mounts in a holder that makes electrical contact through the gold contact pads and is connected to the ECIS instrumentation.

RESULTS

Orbital fibroblasts allowed to proliferate in culture assume an appearance characteristic of most fibroblasts. As the phase-contrast micrographs in Fig. 1 demonstrate, few differences in untreated dermal (Fig. 1*A*) and orbital (Fig. 1*C*) fibroblasts were evident. When the culture medium of orbital fibroblasts from a patient with Graves ophthalmopathy was supplemented with PGE_2 (1 μM), the central nucleus-containing region of the cells became elevated with respect to the substratum and began to form cellular processes within 6 hr of prostanoid addition (Fig. 1*D*). The apparent threshold of this effect of PGE_2 was around 10 nM and was dose dependent. In contrast, dermal fibroblasts failed to exhibit an altered morphology when treated under identical culture conditions (Fig. 1*B*). The changes in the orbital fibroblasts were maintained for several hours; however, the cells began to return to their native shapes. By 24 hr after PGE_2 addition, they had flattened out and their processes became less prominent. This reversion to a morphology similar to that seen in untreated cultures occurred despite the continued presence of PGE_2 and could not be prevented with readdition of fresh prostanoid to the medium.

Orbital fibroblasts emanating from patients with Graves ophthalmopathy were considerably more responsive to this effect of PGE_2 than were those from individuals without

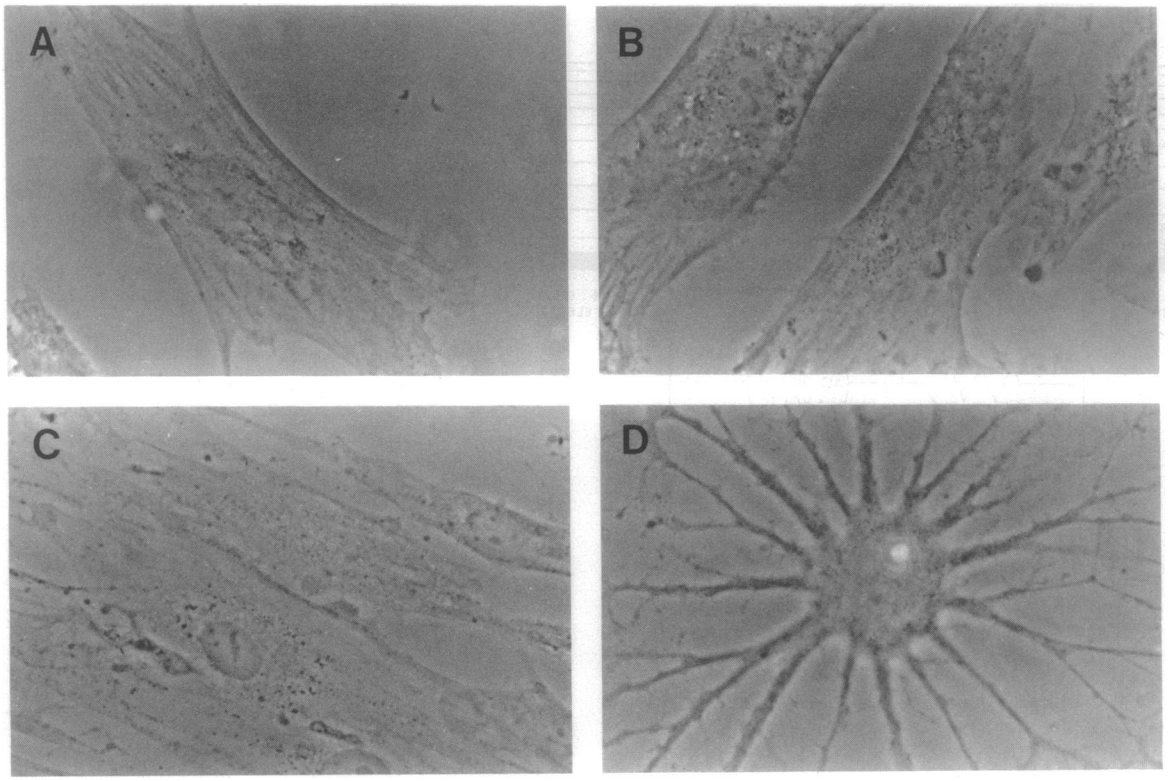

FIG. 1. Phase-contrast micrographs of human dermal (*A* and *B*) and orbital (*C* and *D*) fibroblasts in primary culture. Cultures were inoculated on glass coverslips and allowed to attach for at least 1 day before treatment with PGE_2 (1 μM) (*B* and *D*) for 6 hr or with medium alone (*A* and *C*). (×400.)

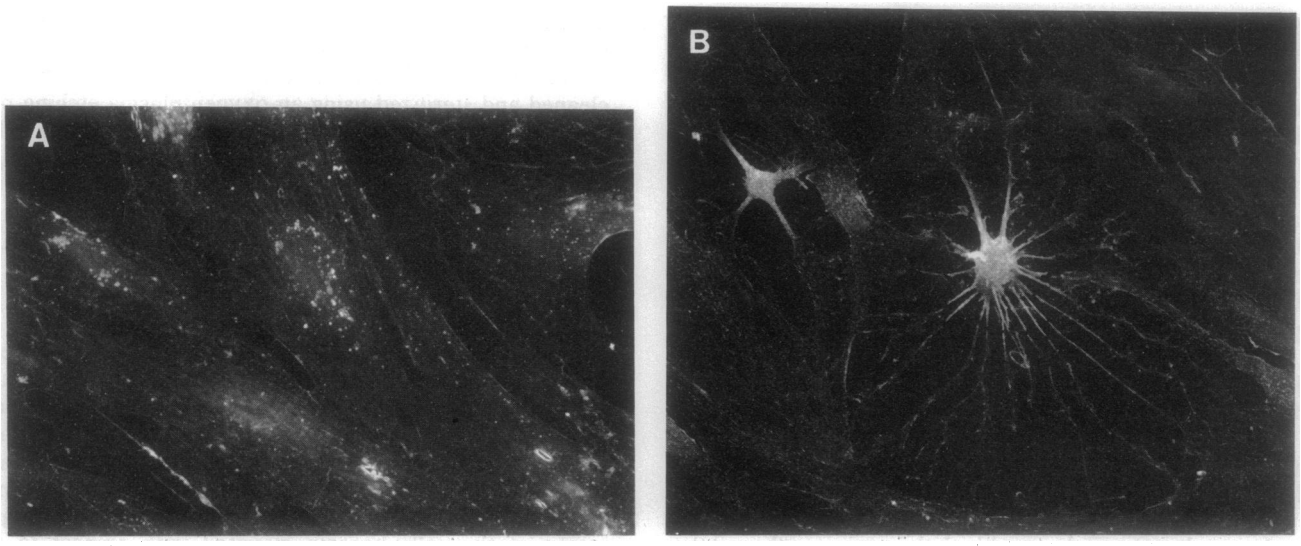

FIG. 2. Scanning electron micrographs of cultured human orbital fibroblasts from a patient with Graves ophthalmopathy. (*A*) Control. (*B*) After treatment for 6 hr with PGE_2 (1 μM). Two arborized cells are seen. (×300.)

orbital disease. Within a given culture, fewer cells from the donors without Graves ophthalmopathy exhibited shape changes. The effect of PGE_2 was not dependent on *de novo* protein synthesis because addition of cycloheximide (10 μg/ml; a concentration that inhibited >90% of protein synthesis) failed to block the changes in morphology. In a given culture strain, the proportion of cells exhibiting a shape change appeared to be greater when the serum concentration in the medium was reduced from 10% to 1%. 8-BrcAMP (1 mM), when added to the culture medium of orbital fibroblasts, elicited a morphological change similar to that observed after PGE_2 treatment (data not shown). Dermal fibroblasts failed to respond to the cyclic nucleotide analogue.

The morphologic changes demonstrated by light microscopy were detailed by scanning electron microscopy. Fig. 2 contains images of control (untreated) orbital fibroblasts (Fig. 2*A*) and those treated for 6 hr with PGE_2 (1 μM; Fig. 2*B*). These cultures emanate from the orbital connective tissue of a patient with Graves ophthalmopathy. Control cells appear flattened with elevated central, nuclear regions. These fibroblasts varied in outline from fusiform to stellate with 3 to 5 broad tapering processes. Other cells were more oval and

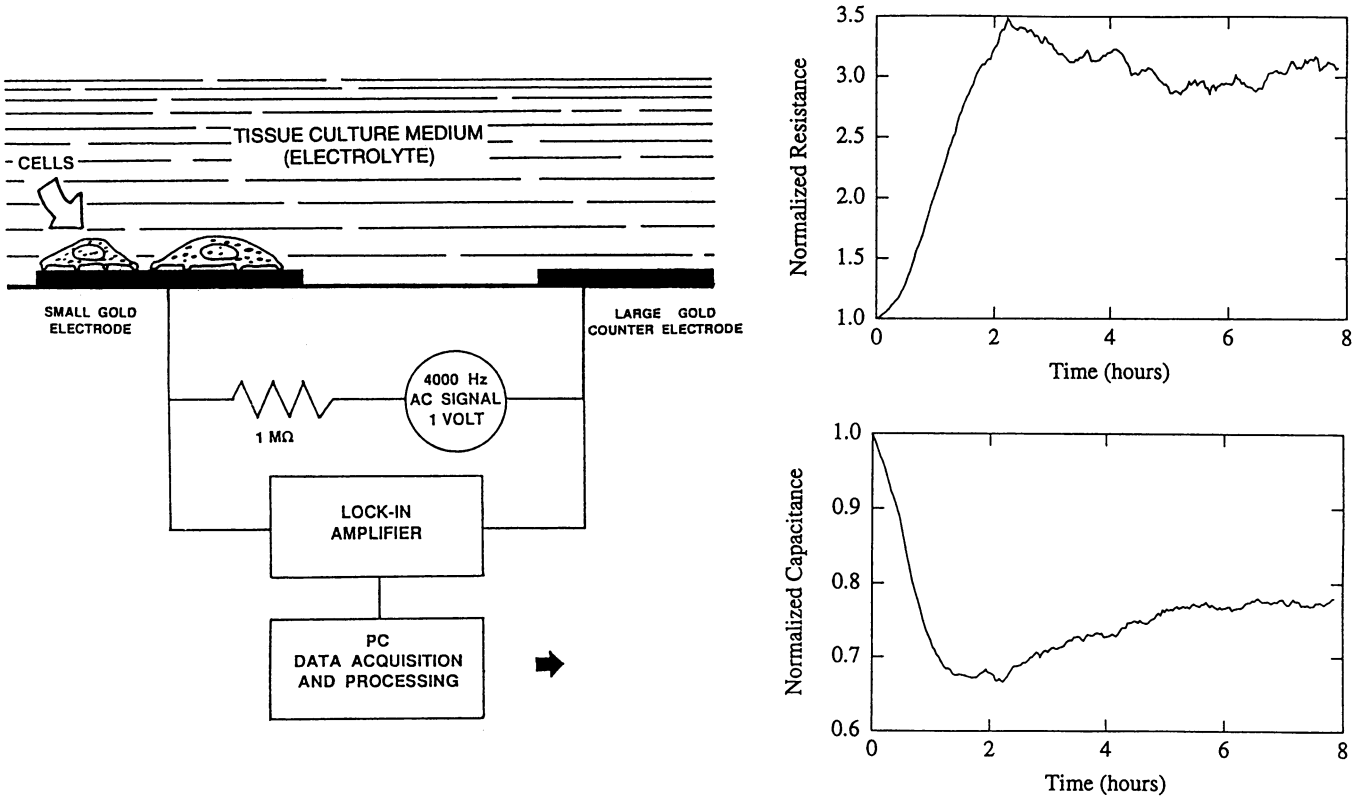

FIG. 3. (*Left*) Schematic of the basic setup to measure the impedance of primary human fibroblast culture monolayers. (*Right*) Data concerning the changes in normalized resistance and capacitance accompanying cell attachment to the substratum. These values are calculated by treating the cell-electrode system arbitrarily as a series resistance–capacitance circuit.

lacked major processes. In contrast, fibroblasts treated with PGE_2 had many slender, radiating processes. The number of processes varied from 3 or 4 to 18–20 per cell. Examination of the distal tips of these processes showed a splaying into broad, irregularly shaped regions that overlapped with similar terminations of the adjacent cells.

In the present study, we used ECIS to assess the kinetics of the shape changes caused by PGE_2. Fig. 3 shows a schematic of the basic setup of electrodes used in these measurements; as the figure demonstrates, tissue culture medium serves as the electrolyte. Fig. 3 contains data pertaining to the changes in capacitance and resistance that accompany cell attachment to the substratum. Capacitance decreased by ≈30% while resistance increased 3-fold within 5 hr after cell inoculation. The levels reached were maintained over the course of data collection (22 hr).

The changes in morphology elicited by PGE_2 in orbital fibroblasts were accompanied by a substantial decrease in monolayer impedance (Fig. 4*A*). The effect is apparent almost immediately and within 30 min of prostanoid addition to the culture medium there was a 10% decrease in normalized resistance. Addition of medium alone did not change the resistance. The impedance continued to fall in the PGE_2-treated culture so that by 4 hr it was 35% below control values. In contrast, monolayer resistance in the untreated culture well remained relatively constant. By 8 hr, resistance in the treated monolayer had begun to return toward initial levels. Addition of PGE_2 to monolayers of dermal fibroblasts failed to alter impedance, as demonstrated in Fig. 4*B*.

DISCUSSION

These studies demonstrate a previously unrecognized ability of orbital fibroblasts from individuals with Graves ophthalmopathy to undergo dramatic morphologic changes *in vitro* in response to concentrations of PGE_2 that have physiological relevance. Alterations in shape elicited by the prostanoid were accompanied by a rapid reduction in cell monolayer impedance, as assessed by ECIS. Results with this noninvasive method correlated well with the microscopic observations of the cell layers and provided quantitative data regarding the dynamics of shape change caused by prostanoid treatment. The electrical method has been shown previously to be highly sensitive to changes in cell morphology (12). The effects of PGE_2 on the impedance measurement were rapid, occurring within a few minutes after its addition to the culture medium. This decrease, reflecting the earliest morphologic alteration in orbital fibroblasts, was imperceptible at the microscopic level until 2 or 3 hr after treatment. Thus, ECIS represents a powerful adjunctive method for assessing the kinetics of subtle shape changes in adherent cells in culture.

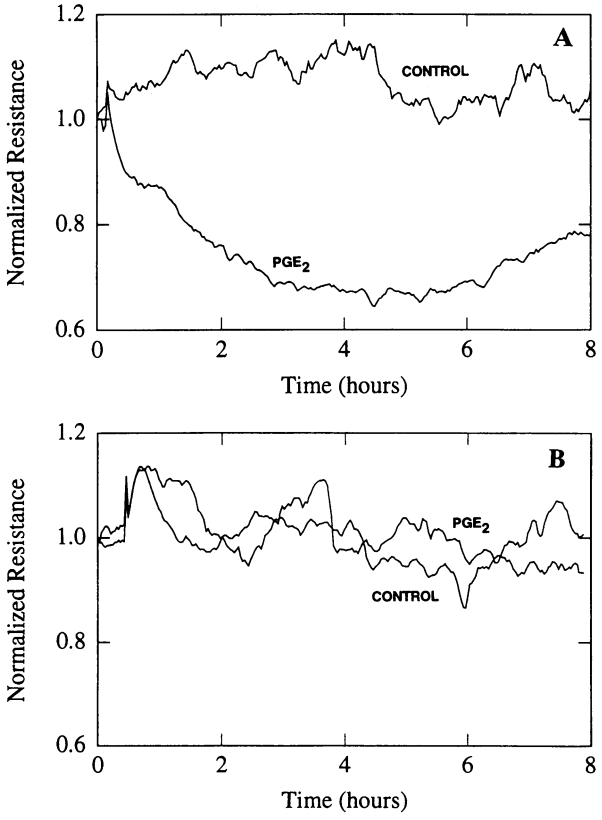

FIG. 4. Effect of PGE_2 on the impedance of orbital (*A*) and dermal (*B*) fibroblasts in primary culture. Cells were allowed to attach to the electrodes as described in *Materials and Methods* for 1 day. Medium vehicle without or with PGE_2 (1 μM, final concentration) was added at time 0.

PGE_2-treated orbital fibroblasts resemble in appearance a number of previously described cell types. Stellate cells found in primary cultures from the synovial membrane of patients with rheumatoid arthritis have a similar morphology (18, 19). Goto and co-workers (20) demonstrated that a subset of synovial cells, also from joints involved with rheumatoid arthritis, exhibited a fibroblast-like morphology during long-term culture but became stellate in response to PGE_2. The concentration of PGE_2 necessary to elicit changes in synovial cell morphology was similar to that used in the current studies. While not characterized further, the effect apparently evolved over 24 hr and there was no mention of any reversion to the native fibroblast morphology. These synovial cells were found to produce an interleukin 1-like factor, which the authors speculated had a role in the pathogenesis of the disease (20). PGE_2 may mediate the morphological changes and actin reorganization associated with the action of epidermal growth factor (21). The stellate appearance of treated orbital fibroblasts is also similar to that of epidermal Langerhans cells and peripheral blood dendritic cells (22, 23). However, we can only speculate currently about what immunological role, if any, orbital fibroblasts might play that is distinct from other populations of fibroblasts. Further investigation into cell lineage, profiles of cytokine production, and immunological markers expressed by orbital fibroblasts that distinguish them from others remains to be done. Recent evidence has been put forward suggesting that neural crest-derived cells display great diversity and plasticity (24, 25). Orbital connective tissue is of neural crest origin (26). Thus, the ability of PGE_2 to elicit the change in orbital fibroblast shape *in vitro* may reflect a fundamental cellular characteristic related to its embryonic derivation.

The mechanism involved in the action of PGE_2 we report here is not known. Prostanoids elicit their diverse cellular actions by associating with plasma membrane receptors (27–29). A number of PGE_2 effects are mediated through the activation of adenylate cyclase (30). The ability of an active cAMP analogue to mimic the PGE_2 effect suggests the possibility that such an activation is involved in the morphologic changes described here. It should be noted that PGE_2 has been shown previously to play an important role in the maintenance of corneal endothelial cell shape in culture (31). This action appears to be mediated through receptors of the EP_2 subtype and is related to the generation of cAMP (32).

Graves ophthalmopathy represents an enigmatic process in which glycosaminoglycans, largely hyaluronan, accumulate in the soft tissues of the orbit (33). While substantial insights have been made into the pathogenesis of the hyperthyroid goiter associated with Graves disease, the events surrounding the anatomic site-selective activation of connective tissue in the orbit remain undefined. The infiltration of the extraocular musculature and fibroadipose tissue with T lymphocytes (34, 35) suggests a pathogenic role for cellular immunity;

however, the question of why orbital tissue should manifest this systemic disease is not yet resolved. Our results define a phenotypic attribute of cultured orbital fibroblasts from patients with Graves ophthalmopathy that sets them apart from other types of fibroblasts. Evidence that these cells may participate in the pathogenesis of the disease derives mainly from their ability to selectively respond to interferon γ in regard to an upregulation in the accumulation of glycosaminoglycans, an action not observed in dermal fibroblasts (8). We have recently found that leukoregulin, another lymphokine (36), can also enhance the synthetic rate of hyaluronan in orbital fibroblasts. It is of interest that leukoregulin can stimulate the production of PGE_2 in orbital fibroblasts, resulting in an altered cellular morphology identical to that described here (unpublished observations). Whether these responses to PGE_2 are relevant to the normal function of orbital connective tissue or to any disease state remains to be determined.

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