Chapter 24

DEVELOPMENT OF A FIBROBLAST PROLIFERATION BIOASSAY TO DETECT MEDIATORS OF PULMONARY FIBROSIS

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INTRODUCTION

Proliferation and enhanced synthesis of collagen by pulmonary fibroblasts have been shown to be key steps in the development of chronic silicosis (Goldstein and Fine, 1986). The regulation of lung fibroblast proliferation by cytokines released from alveolar macrophages may be an important pathogenetic mechanism in the development of the fibrotic process (Kelley, 1990). One cytokine, platelet-derived growth factor (PDGF), promotes fibroblast proliferation by inducing the movement of quiescent (Go) cells into the G1 phase of the cell cycle (Chen and Rabinovitch, 1989). Others regulate the rate of transition of fibroblasts from G1 into the S phase (Leof et al., 1982). These two classes of cytokines have been termed, respectively, competence and progression factors. One approach used to examine the release of cytokines from macrophages is the fibroblast proliferation assay in which fibroblasts are exposed to culture supernatants from macrophages exposed to various stimuli. In most of these assays, the supernatant contains fetal calf serum which provides the competence factor(s) necessary to facilitate the proliferation of fibroblasts (Bitterman et al., 1982; Bitterman et al., 1983; Elias et al., 1988). Recently, a fibroblast proliferation assay using platelet-poor plasma (lacking competence factor(s)) as a substitute for fetal calf serum has been described (Kuman et al., 1988; Bauman et al., 1990). In this assay, the release of a competence-inducing PDGF-like growth factor from rat and human macrophages can be distinguished from other cytokines that act as progression factors. In order to obtain more consistent results and with the ultimate goal to be able to discriminate between the effects of competence factors as opposed to progression factors, we have conducted experiments to determine the appropriate concentrations of plasma and PDGF required for imparting competence in the fibroblast proliferation assay. We tested lung fibroblast cells obtained from explants of rat lung tissue and also a fetal human lung fibroblast cell line obtained from American Tissue Culture Collection (ATCC153).

MATERIALS AND METHODS

Fibroblasts

Specific pathogen-free, male Sprague-Dawley rats were use in some studies. Animals were given a lethal intraperitoneal dose of sodium pentobarbital. Fibroblasts were isolated by chopping the lung in enzymes that digest the connective tissue but liberate lung cells for further study (Rabovsky et al., 1989). After digestion, the remaining lung tissue suspension was filtered through two layers of sterile gauze and centrifuged to recover lung fibroblasts. These were resuspended in culture medium that contained 10% fetal calf serum and distributed to culture plates for growth. In other experiments, a human fetal lung fibroblast cell line, obtained from American Type Culture Collection, Rockville, MD, 20852, was used instead of rat lung fibroblasts. In these cases, a 1 ml ampule containing human fetal fibroblasts was plated into a tissue culture flask containing medium plus 10% fetal calf serum. For both types of fibroblasts, culture medium was changed 3 times per week and cultures were incubated at 37°C until confluent. Harvested rat and human lung fibroblasts were quantified using an electronic cell counter equipped with a cell sizing attachment (Coulter Electronics, Inc., Hialeah, Florida).

Tritiated Thymidine Incorporation

The basic procedural outline of Kumar et al. (1988) was used with modifications to evaluate tritiated thymidine incorporation into fibroblast DNA following exposure to PDGF and plasma. Both rat and human lung fibroblasts were plated at 50,000 cells/ml at a density of 250,000 cells/25cm^2 culture plate. Cells were quiesced for 4 days with 2% rat plasma. As the assay was refined, fibroblasts were quiesced in plasma-free media for 48 hrs, since the mitogenic activity of 2% plasma was variable. Test medium was applied for a period of 6 hrs, followed by a 24 hr tritiated thymidine (1μCi/ml) labelling period in plasma-free media. Medium alone was used as a negative control and media with 10 or 20% fetal calf serum was used as the positive control for rat and human fibroblasts, respectively.

Cell Quantification and Measurement of Mitogenesis

Twenty-four hours after the addition of tritiated thymidine, the fibroblasts were washed with 5ml of fresh serum-free media, centrifuged and resuspended in phosphate-buffered saline. The cells were dissolved in 0.5ml of 0.1N NaOH and radioactivity determined in a beta counter. Incorporation of trititaed thymidine as an index of DNA synthesis was expressed as DPM/fibroblast.

RESULTS

In the present study, we quantified mitogenic potential by monitoring the incorporation of tritiated thymidine as

dpm/cell. Figure 1 shows data with results as predicted by Kumar et al (1988).

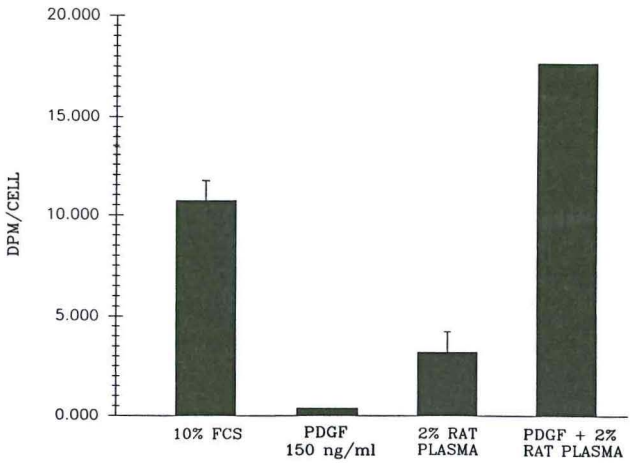

FIGURE 1

Figure 1. [3H]thymidine incorporation (24 hrs) in rat fibroblasts quiesced in 2% rat plasma for 4 days and exposed to test medium (150ng/ml PDGF, 2% (v/v) rat plasma, or PDGF plus 2% rat plasma) for 6 hrs. MEM plus 10% fetal calf serum served as the positive control. Values for fetal calf serum and plasma are means ± standard errors of two experiments while data for PDGF are from a single experiment.

The addition of competence factor (150ng/ml PDGF) or progression factor (2% rat plasma) alone did not result in significant mitogenesis relative to the positive control, i.e., 10% serum which contained both competence and progression factors. The combination of PDGF and 2% rat plasma did result in activation of mitogenesis which in fact exceeded the positive control. This figure is part of a larger experiment which looked at concentrations of rat plasma above 2% to determine if additional activation could be generated by the higher plasma concentrations. Higher concentrations of plasma did not result in significantly higher activation (data not shown).

Figure 2 is PDGF dose response curve which defined the practical limits of this assay for detecting the competence stimulating activity and, hence, mitogenic activity of mediators potentially released from particle-stimulated alveolar macrophages. These results suggest that the assay is effective down to a PDGF concentration of 25ng/ml.

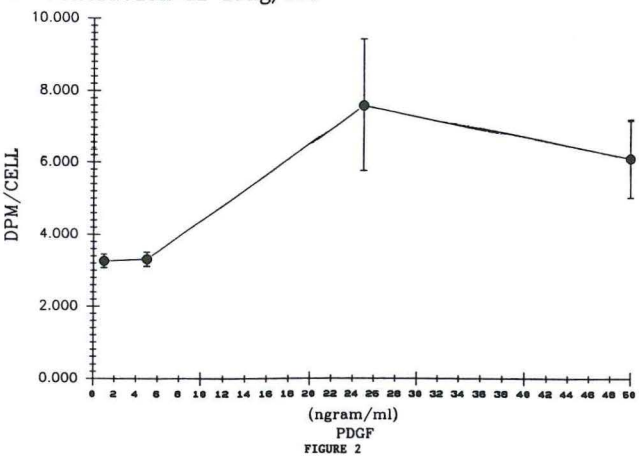

FIGURE 2

Figure 2. PDGF dose response for [3H]thymidine incorporation in human fetal lung fibroblasts quiesced in plasma-free media and exposed concurrently to 1% human plasma. Values are means ± standard errors of two experiments.

DISCUSSION

Our goal was to refine existing in vitro assays which have been used to evaluate the fibrogenic potential of respirable dusts frequently encountered within occupational settings. Alveolar macrophage-derived growth factor (AMDGF), a PDGF analog, is known to be released from macrophages in response to dust exposure (Bitterman et al., 1982; Bitterman et al., 1983; Bitterman et al., 1986). Alveolar macrophages, exposed to dusts in vivo or in vitro, produce AMDGF rich media in culture which has been applied to fibroblasts and tested for mitogenic and fibrogenic potential (Bitterman et al., 1982; Kumar et al., 1988; Leibovich and Ross, 1976).

Earlier studies by Kumar and et al (1988) demonstrated that the percentage of nuclei labelled with tritiated thymidine increased significantly when 2% platelet-poor rabbit plasma and PDGF were provided concurrently to quiesced rat fibroblast cultures. Similar results using dpm/cell as a measure of mitogenicity were obtained in our laboratory with the substitution of 2% plasma obtained from rats. In addition to evaluating the dose-dependent competence activity of plasma (needed solely for its progression activity), an alternative method to eliminate the problem of competence altogether would be to use media characterized by the addition of specific progression factors. The final question to be raised is one of the sensitivity of the bioassay for PDGF. In other words, how much competence activity must be present in the applied supernatant to cause a measurable mitogenic response. Results indicate that the assay is sensitive at PDGF levels as low as 25ng/ml. Kumar et al (1988) have estimated that 1-3.5ng are released per million alveolar macrophages per 24 hr period. This would suggest that employing our assay conditioned media from 7.14 million alveolar macrophages would be sufficient to demonstrate dust-induced secretion of mitogenic cytokines.

In summary, the results of this study represent important refinements of published methods for a bioassay of fibrogenic factor production.

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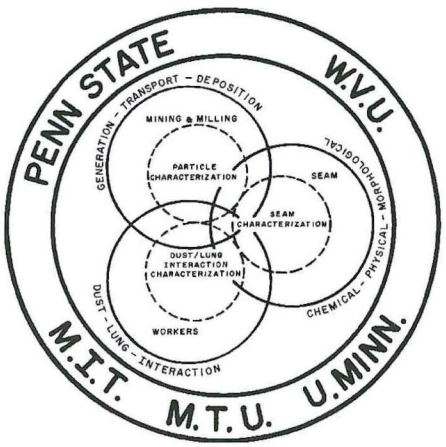

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