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LIVER FIBROBLAST PROLIFERATION IN MURINE SCHISTOSOMIASIS

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Abstract. Liver fibrosis in schistosomiasis is associated with prominent accumulations of fibroblasts. Primary cell cultures were prepared from the fibrotic livers of *Schistosoma mansoni*-infected mice, and cells with the appearance of fibroblasts by light microscopy were isolated from these cultures. Proliferation of these cells was examined in coculture experiments with syngeneic inflammatory cells. T cell-enriched mononuclear cells from spleens of *S. mansoni*-infected or normal mice, and Kupffer cell/macrophages from fibrotic liver all stimulated the proliferation of liver fibroblasts, as measured by ^{3}H-thymidine uptake. Primary cultures of mouse skin fibroblasts showed similar responses to coculture, but an established fibroblast line, 3T3, was unresponsive. Cell-free supernatant medium from coculture experiments did not affect fibroblast proliferation, perhaps because of the requirement for serum in the culture medium. Liver fibroblasts derived from this disease model may be especially suitable for study of the interaction between tissue inflammation and fibrosis.

Hepatic fibrosis may result from the proliferation of collagen-synthesizing cells, from increased formation of collagen in existing cells, or from deficient collagen degradation in the face of continuing collagen formation.[1] Mice infected with *Schistosoma mansoni* are a useful model system for the study of hepatic fibrosis.[2] In this system, schistosome egg antigens elicit the formation of granulomas, followed by proliferation of fibroblasts or fibroblast-like cells and deposition of collagen associated with the granulomas.[3,4]

At least two factors appear to be involved in fibroblast proliferation in murine schistosomiasis. Schistosome egg granulomas contain a soluble material that stimulates proliferation of dermal fibroblasts,[5–7] and schistosome eggs themselves contain a different material, as assessed by molecular size and charge, that also stimulates dermal fibroblast proliferation.[8] In addition to proliferation, fibroblast chemotaxis and collagen production are stimulated by products of granulomas and eggs, respectively.[9,10]

The nature of the cells involved in collagen formation in the liver is not well defined. Fibroblast-like cells with characteristics of classical fibroblast, myofibroblasts, and perisinusoidal lipocytes all synthesize collagen and have been implicated in liver fibrosis.[1] These cells may not have the same responses as dermal fibroblasts to various stimuli.

Direct contact between fibroblasts and inflammatory cells may be important for fibroblast stimulation. In a study of stimulation of collagen formation by human skin and lung fibroblasts, coculture with mononuclear cells was a more potent stimulant than the addition of cell-free culture supernatant fluids to the fibroblasts.[11]

In order to study the proliferation of liver fibroblasts or fibroblast-like cells in murine schistosomiasis, we prepared primary cultures of cells from *S. mansoni*-infected mouse liver, and isolated collagen-synthesizing cells with the appearance of fibroblasts by light microscopy. We examined their proliferation in response to coculture with syngeneic mononuclear cells, and compared this response to that of other fibroblasts of nonhepatic origin.

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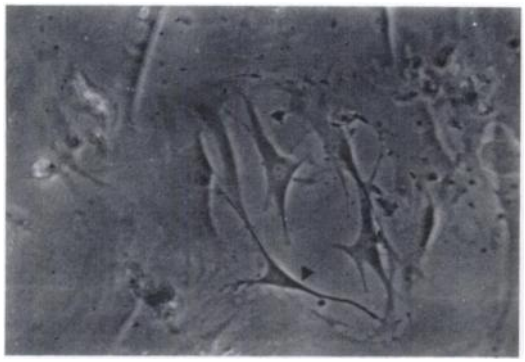

FIGURE 1. Primary culture from a cell suspension prepared by enzymatic digestion of an *S. mansoni*-infected mouse liver, as described in Materials and Methods. After 10 days in culture, the 2 predominant cell types are pale cells with the appearance of fibroblasts, and cells containing refractile cytoplasm with the appearance of Kupffer cells or macrophages (arrow). Phase contrast, original magnification ×200.

FIGURE 2. Isolated liver fibroblasts. Fibroblasts were separated from primary cultures by trypsinization as described in Materials and Methods. Phase contrast, original magnification ×200.

MATERIALS AND METHODS

Mouse liver fibroblast preparation

Female BALB/c mice from our laboratory colony were infected with 100 *S. mansoni* cercariae by subcutaneous injection at 5 to 6 weeks of age. Nine to 10 weeks later, mice were killed by cervical dislocation, livers were removed aseptically, and were perfused free of blood with a sterile solution of 0.03% EDTA in 0.01 M phosphate buffered saline (PBS), pH 7.5, at 25°C. After mincing to 0.5–1 mm^3 pieces with razor blades, livers were enzymatically dissociated in a 125 ml trypsinizing flask at 37°C for 30 min using a sterile solution of trypsin, clostridial collagenase, hyaluronidase, and DNAase prepared as described by Wu et al., using the same sources of materials.[12] Two to 3 livers, total weight 5–9 g, were dissociated in 50 ml of enzyme solution. The resulting cell suspension was pelleted at 800 × *g* for 10 min at 25°C and washed once in Eagle's basal medium supplemented with 10% fetal calf serum, 0.2 mM L-proline, 50 mg/ml ascorbic acid, 50 μM ferrous ammonium sulfate, 25 mM HEPES, gentamicin 0.1 mg/ml (Schering Co., Manati, Puerto Rico), and mycostatin 50 U/ml (Gibco, Grand Island, New York).

The washed cell suspensions were placed in 25-cm^2 culture flasks using 10 flasks per liver, and 5 ml of the same medium per flask. Medium was changed after incubation for 24 hr at 37°C and incubation was continued for 8 to 10 days. Over this time, 2 predominant cell populations remained viable and adherent to the plastic surface. One cell population had refractile cytoplasm as observed by phase contrast microscopy, with short cytoplasmic processes, and resembled Kupffer cells or macrophages (Fig. 1). The other predominant cell type had nonrefractile cytoplasm, longer projections, and resembled fibroblasts by light microscopy (Fig. 1). Trypsin, 0.2%, and 0.03% EDTA in 0.01 M PBS, pH 7.5, were added to cell cultures for 10 min at 37°C resulting in the continued adherence of the Kupffer cell/macrophage population to the flask surface and dissociation of the fibroblast-like population. The dissociated cells, referred to hereafter as liver fibroblasts, were pelleted and washed once in medium as described above, counted with a hematocytometer, and transferred to new 25-cm^2 flasks at a density of 2×10^5 cells/flask, with a plating efficiency of 50%. Purity of the liver fibroblast population prepared in this manner was between 90% and 95% as judged by light microscopy (Fig. 2).

Preparation of other fibroblasts

Skin from 1–2-week-old female BALB/c mice was minced, dissociated, and cultured as described for liver cell suspensions above. Skin fibroblasts were dissociated from primary cultures and transferred to new flasks at a density of 2×10^5 cells per flask. A BALB/c-derived fibroblast cell line, 3T3, preserved in its 113th passage in liquid nitrogen (American Type Culture Collection, Rockville, Maryland) was thawed, washed

in medium, cultured for 10 days, trypsinized, and placed into new culture flasks at the same density as liver and skin fibroblasts.

Preparation of spleen and thymus cells

Spleens were removed from *S. mansoni*-infected female BALB/c mice 10 weeks after infection, and from uninfected mice. Thymi were removed from 1–2-week-old female BALB/c mice as well. After teasing the tissue in culture medium with a rubber policeman, cell suspensions were counted and T-cell enriched nonadherent cells were separated by elution from a column of nylon wool (Type 200, Fenwal Laboratories, Deerfield, Illinois) after 45 min incubation at 37°C.[13] Cells were >80% viable as assessed by Trypan blue exclusion.

Coculture of fibroblasts with mononuclear cells

Cultures of fibroblasts were combined with T-enriched nonadherent spleen and thymus cells 1 day after preparation of the fibroblast cultures, on the same day as preparation of the mononuclear cells. In preliminary experiments, a coculture period of 72 hr with a cell ratio of 100 mononuclear cells per fibroblast (10^7 mononuclear cells: 10^5 fibroblasts per flask) was determined to be optimal for showing differences between control and cocultured fibroblasts, and was used routinely thereafter. Figure 3 shows such a coculture.

Fibroblasts, with or without spleen cells, were also cocultured with flasks of washed trypsin-resistant Kupffer cell/macrophages. The ratio of fibroblasts to Kupffer cell/macrophages was approximately 1:1 by microscopic inspection.

Measurement of fibroblast proliferation

After coculture, flasks were rinsed 3 times with 0.01 M PBS, pH 7.5, with removal of all nonadherent cells by microscopic inspection. Five ml of culture medium with 1.0 μCi/ml (methyl ^{3}H)-thymidine, 2 Ci/mmole (Amersham International, Amersham, United Kingdom), was then added and flasks were incubated for 16 hr, at 37°C. The cells were then trypsinized as described above, using 1.0 ml solution per flask. In preliminary experiments, a 0.1 ml aliquot of cell suspensions was saved for direct cell counting with a hematocytometer. Direct cell counts were found to correlate with ^{3}H-thymidine uptakes. Cell suspensions were harvested on glass fiber filter paper using a multiple automated sample harvester (Brandel Co., Gaithersburg, Maryland). Radioactivity was determined in Kodak Ready-To-Use III scintillation fluid at an efficiency of 37% in a Delta 300 liquid scintillation counter (Tracor Analytic, Elk Grove Village, Illinois). Proliferation was expressed as the mean number of counts per minute (cpm) per 10^5 fibroblasts per flask, ±1 SD, for triplicate determinations. Significance of differences between means was assessed using Fisher's *t*-test on unpaired data.[14]

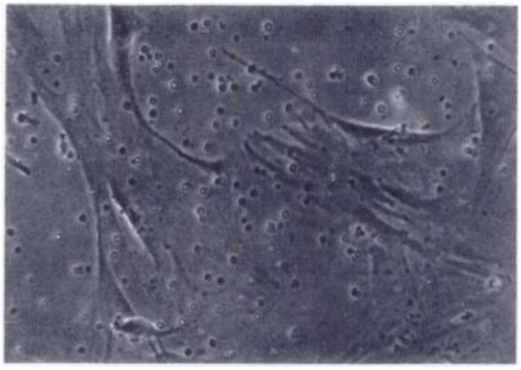

FIGURE 3. Coculture of liver fibroblasts with T-enriched nonadherent spleen cells. After 3 days of coculture, spleen cells were removed and fibroblast proliferation was measured as described in Materials and Methods. Phase contrast, original magnification ×200.

Fibroblast proliferation was also measured after coculture with T-enriched mononuclear cells treated with mitomycin C. Cells from spleens of *S. mansoni*-infected mice were prepared as described above, suspended at a density of 5×10^6/ml in medium containing 25 μg/ml mitomycin C (Sigma), and incubated with frequent rotation for 30 min at 37°C. After 3 washes in medium, the cells were used for coculture with fibroblasts.

Fibroblasts were also incubated with cell-free supernatant conditioned medium from cultures of primary liver cell suspensions and from cocultures of fibroblasts with T-enriched spleen cells. Concentrations of conditioned medium varying from 10% to 80% were used for periods from 1 to 3 days, followed by measurement of proliferation as described above.

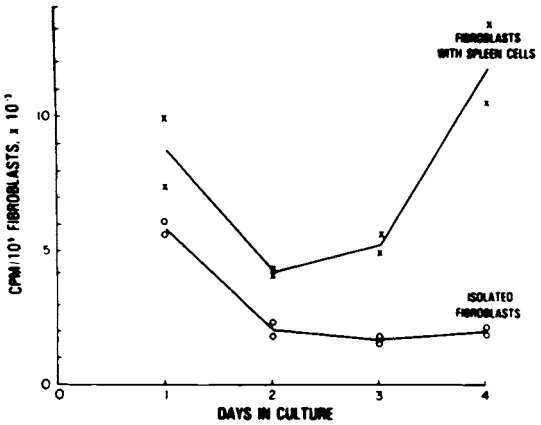

FIGURE 4. Proliferation of isolated liver fibroblasts and liver fibroblasts cocultured with T-enriched spleen cells from *S. mansoni*-infected mice. Spleen cells were added 1 day after isolation of fibroblasts, and fibroblast proliferation was measured after 1, 2, or 3 days of coculture as described in Materials and Methods.

TABLE 1

Coculture of liver fibroblasts with T-enriched mononuclear cells

cpm × 10^{-3}/flask			
Fibroblasts	Fibroblasts + infected spleen cells	Fibroblasts + normal spleen cells	Fibroblasts + normal thymus cells
1.1 ± 0.1	9.7 ± 2.5	6.3 ± 0.8	3.4 ± 1.2

Mean of triplicate determinations ±1 SD.

Fibroblasts were cocultured with T-enriched mononuclear cells of various origins for 3 days and proliferation was measured as described in Materials and Methods. Each flask contained 10^5 fibroblasts at the start of coculture.

Fibroblast proliferation of all cocultures was greater than that of fibroblasts alone ($P < 0.01$). Fibroblast proliferation with infected or normal spleen cells was greater than that with thymus cells ($P < 0.05$).

Similar results were obtained in 2 replicates of this experiment.

RESULTS

Proliferation of liver fibroblasts after dissociation from primary culture is shown in Figure 4. Proliferative activity declined as isolated fibroblasts were maintained in culture. This decline was reversed by coculture with T-enriched spleen cells from *S. mansoni*-infected mice. The maximum difference between proliferation of isolated and cocultured fibroblasts was apparent after 3 days of coculture.

The effect of coculture of liver fibroblasts with T-enriched mononuclear cells from 3 sources is shown in Table 1. Syngeneic cells from the spleens of *S. mansoni*-infected mice and normal mice, as well as thymus cells from 1–2-week-old normal mice, all stimulated fibroblast proliferation. Proliferation of fibroblasts cocultured with spleen cells was greater than that of fibroblasts cocultured with thymus cells. Spleen cells from *S. mansoni*-infected mice caused slightly more stimulation than normal spleen cells, but this difference was not significant.

Coculture of liver fibroblasts with T-enriched infected spleen cells and Kupffer cell/macrophages is shown in Table 2. There was stimulation of fibroblast proliferation from coculture with either cell type. Stimulation with either infected or normal spleen cells was more effective than that with Kupffer cell/macrophages, and combined coculture of fibroblasts with both cell types did not further increase proliferation. Control cultures of Kupffer cell/macrophages with or without spleen cells showed negligible ^{3}H-thymidine uptake.

The effect of coculture on proliferation of syngeneic fibroblasts from 3 sources is shown in Table 3. Both liver and skin fibroblasts isolated from primary culture showed comparable responses to coculture with T-enriched spleen cells and Kupffer cell/macrophages. The 3T3 fibroblasts were unaffected by coculture with either cell type.

In experiments with liver fibroblasts cocultured with mitomycin C-treated T-enriched spleen cells, stimulation of fibroblast proliferation was no different with untreated or mitomycin C-treated spleen cells (data not shown). Multiple experiments with liver fibroblasts incubated in a wide range of concentrations of conditioned medium from cocultures or primary cultures showed no stimulation of fibroblast proliferation.

DISCUSSION

Interaction between inflammatory cells and fibroblasts may be a key mechanism leading to fibrosis. Fibroblasts or fibroblast-like cells may show major functional differences depending on origin and culture conditions.[1] Previous studies of fibroblast functions such as proliferation and collagen formation in hepatic schistosomiasis have used nonhepatic fibroblasts as target cells.[5–10] The present study describes proliferative responses of fibroblast-like cells of hepatic origin. They show comparable responses to those of primary skin fibroblasts in coculture with inflammatory cells, and differ considerably from

TABLE 2

Coculture of liver fibroblasts with T-enriched mononuclear cells and Kupffer cell/macrophages from S. mansoni*-infected mice*

	cpm × 10^{-3}/flask				
Fibroblasts	Fibroblasts + Kupffer cells	Fibroblasts + spleen cells	Fibroblasts + spleen cells + Kupffer cells	Kupffer cells	Spleen cells + Kupffer cells
14 ± 2.0	24.0 ± 2.0*	52.3 ± 9.3**	40.3 ± 5.5**	0.2 ± 0.1†	0.2 ± 0.05†

Means of triplicate determinations ±1 SD.
Fibroblasts were cocultured with T-enriched spleen cells, Kupffer cell/macrophages, or both for 3 days and proliferation was measured as described in Materials and Methods. Each flask contained 10^5 fibroblasts at the start of coculture.
* Greater than proliferation of fibroblasts alone ($P < 0.01$).
** Greater than proliferation of fibroblasts alone ($P < 0.01$) or fibroblasts with Kupffer cell/macrophages ($P < 0.02$).
† Less than proliferation of any culture containing fibroblasts ($P < 0.001$).
Proliferation of fibroblasts cocultured with spleen cells was not affected by addition of Kupffer cell/macrophages (no significant difference).
Similar results were obtained in 3 replicates of this experiment.

an established fibroblast line which was not affected by the same coculture conditions.

In this study, liver fibroblasts were cocultured at a preconfluent cell density. Cocultures of postconfluent fibroblasts were not used for studies of proliferation, although they might be suitable for examination of collagen synthesis or other fibroblast functions. The optimal ratio of 100 T-enriched mononuclear cells per fibroblast for this study was determined empirically. It was not possible to vary the ratio of Kupffer cell/macrophages to fibroblasts in the same manner, because of their strong adherence to culture flasks. The consistently greater stimulation produced with T-enriched mononuclear cell cocultures compared to that of Kupffer cell/macrophage cocultures is probably a reflection of this factor. No synergistic effect was observed when fibroblasts were cocultured with both cell types together.

In all experiments, the degree of stimulation with coculture ranged from 2- to 10-fold increases in proliferation compared with that of control fibroblasts. Radioactivity measurements were internally consistent in all experiments, but were not comparable between different experiments. For example, there was a >10-fold difference between the radioactivity measurements for control fibroblasts in the experiments reported in Table 1 and Table 2. There were no differences between such cultures in microscopic appearance or direct counts of cells. We were unable to measure the intracellular pools of nonradioactive thymidine in these cells. Dilution of radioactive amounts of cold thymidine could explain the radioactivity differences between different experiments, with internal consistency for all flasks derived from the same fibroblast preparation.

The proliferative activity measured in these experiments may be attributed almost completely to fibroblasts even though other cell populations were present. Complete removal of the nonadherent T-enriched mononuclear cells was assured by inspection between addition of ra-

TABLE 3

Coculture of fibroblasts from primary liver, primary skin, and established cell line sources with T-enriched mononuclear cells and Kupffer cell/macrophages from S. mansoni*-infected mice*

	cpm × 10^{-3}/10^5 fibroblasts			
Fibroblast source	Fibroblasts	Fibroblasts + Kupffer cells	Fibroblasts + spleen cells	Fibroblasts + spleen cells + Kupffer cells
Liver	9.3 ± 0.6	18.7 ± 1.5*	40.0 ± 8.0**	30.7 ± 2.5**
Skin	5.9 ± 0.9	12.6 ± 2.5*	18.7 ± 2.1**	22.0 ± 3.0**
3T3	26.3 ± 3.4	19.3 ± 4.2†	20.6 ± 4.5†	27.0 ± 4.6†

Means of triplicate determinations ±1 SD.
Fibroblasts were cocultured with T-enriched spleen cells, Kupffer cell/macrophages, or both for 3 days and proliferation was measured as described in Materials and Methods.
* Greater than proliferation of fibroblasts alone ($P < 0.02$).
** Greater than proliferation of fibroblasts alone ($P < 0.01$) or fibroblasts with Kupffer cell/macrophages ($P < 0.05$).
† Proliferation not affected by coculture (no significant difference).
Proliferation of fibroblasts cocultured with spleen cells was not affected by addition of Kupffer cell/macrophages (no significant difference).
Similar results were obtained in 3 replicates of this experiment.

dioactive thymidine. Kupffer cell/macrophages showed negligible thymidine uptake even after coculture with spleen cells. Lack of effect of mitomycin C treatment on the stimulation caused by T-enriched mononuclear cells suggests that proliferation of the stimulating cells themselves during the coculture period is not a factor in eliciting the observed effects on fibroblasts.

We found no effect of cell-free conditioned medium on fibroblast proliferation in this system. One possible explanation for this result is the requirement for serum-containing medium to support the primary culture-derived fibroblasts. Attempts to adapt these cells to serum-free medium, with or without added fibronectin, resulted in rapid loss of viability. Most studies of fibroblast-stimulating factors in schistosomiasis and other diseases require serum-free medium to show an effect.[5-9, 15]

Coculture experiments, with their potential for direct contact between fibroblasts and inflammatory cells, may be more sensitive for showing effects on fibroblasts than experiments with soluble mediators, as pointed out by Hibbs et al.[11] They suggested that fibroblast proliferation or other functions might be affected by direct stimulation of fibroblasts by mononuclear cells, or that cell-cell contact might increase the sensitivity of fibroblasts to the effects of soluble factors. Other studies of cocultures of mononuclear cells and fibroblasts support the potential importance of direct cell-cell contact for modifying functions of both cell populations.[16, 17]

The fibroblast-like cells derived from *S. mansoni*-infected mouse liver which were examined in this study had the light microscopic appearance of fibroblasts and were shown to actively synthesize collagen in studies not reported here. These cells could have originated from a number of cell types in response to the stimulus of inflammation and granuloma formation in the liver, as discussed earlier. In this regard, we were unable to obtain usable numbers of fibroblast-like cells from normal mouse liver using the same methods. The cells derived from this disease model may be especially suitable for study of the interaction between tissue inflammation and fibrosis.

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