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In vitro studies on extracellular matrix production by *M. leprae* infected murine neurofibroblasts

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Summary Fibroblasts and a host of macrophage secretory products have been implicated in a number of diseases where excess extracellular matrix (ECM) deposition is the main pathological feature. Fibrosis characterized by excessive deposition of collagen also contributes to the irreversible nerve damage observed in leprosy. Since *M. leprae* are seen within neurofibroblasts (Nf) in the advanced stages of the disease and macrophages form a common infiltrating cellular constituent of leprous nerves at all stages, secretion of ECM proteins by Nf was studied, *in vitro* following infection with *M. leprae* and in the presence of macrophage secretory products. These studies were compared in cells derived from two strains of mice, Swiss White (SW) and C57BL/6, as they differ in their response to *M. leprae* infection and parallel those observed in lepromatous and tuberculoid patients, respectively. On infection with *M. leprae*, Nfs showed a decrease in secretion of collagen type IV in SW and type I in C57Bl/6 strain. Macrophages caused a further decrease in the secretion of collagen types affected by *M. leprae* infection *per se*, while the other collagen types, viz. I and III in SW strain and III and IV in C57Bl/s strain, were unaffected. This study indicates that neural collagenization in nerves in advanced leprosy may be of Nf origin. However, unlike other diseases with excess collagen deposition, ECM proteins produced by Nfs in response to nerve damage may not be of prime importance in the progression of leprous neuropathy and occur as a general response to loss of cellular content in leprous nerves.

Introduction

Neurofibroblasts (Nfs) form a major portion of the non-neuronal cell population that is present in the peripheral nerve. They are present scattered throughout the epineurium and endoneurium,[1] and cells forming the perineurium have also been shown to be of fibroblast origin.[2] In conjunction with Schwann cells, fibroblasts provide the extracellular matrix (ECM) components of the nerve,[3,4] which consists prominently of collagen and fibronectin.

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Fibroblasts are actively involved in general tissue repair, during which they proliferate and secrete ECM proteins, proteases and protease inhibitors.[5–7] Following nerve injury, the neural fibroblast population increases, and recent work indicates that along with macrophages, they may also be important in the removal and reutilization of myelin debris due to their ability to express apolipoproteins.[8] Their importance in nerve repair is further highlighted by their ability to reinstate the competence of dystrophic mouse-derived Schwann cells to produce and assemble normal basal lamina,[9,10] while ECM proteins produced by them are known to induce neurite outgrowth.[11] Like other tissues, peripheral nerve repair is also aided by macrophages,[12] and a number of macrophage factors have been shown to regulate ECM metabolism in fibroblasts. However, fibroblasts and macrophages have also been implicated in the initiation and progression of a number of diseases where excess ECM deposition is the main pathological feature.[13]

Fibrosis characterized by excessive deposition of collagen, and extensive proliferation of Nfs contributes to the irreversibility of leprous nerve damage.[14] Histopathological observations show evidence of active ECM accumulation in both early and late leprous nerve.[15,16] However, in the early stages, *M. leprae* is seen only within Schwann cells. In addition, in the early stages, fresh collagen pockets are observed around involved fibres[17] and *in vitro* studies demonstrate an increase in collagen production by *M. leprae*-infected Schwann cells.[18] This indicates perturbed Schwann cell ECM metabolism as the contributing factor at this stage of the disease. It is only in the later stages of nerve damage, when a general increase in the interstitial collagen is observed, that *M. leprae* are seen within fibroblasts. This, in addition to the fact that macrophages are the common infiltrating cellular constituent of the leprous nerve, indicates a strong possibility for *M. leprae* and macrophage mediated and regulated accumulation of ECM proteins by Nfs. Therefore ECM protein secretion by Nf cultures was studied following infection with *M. leprae* and in the presence of macrophage secretory products, to determine the contribution of these cells in leprous nerve fibrosis and collagenization. These studies were compared in cells derived from two strains of mice, Swiss White (SW) and C57BL/6, as they differ in their nerve pathology,[19] Schwann cell[18,20,21] and macrophage functions[22] in response to *M. leprae* infection. The macrophage responses to *M. leprae* infection in SW and C57Bl/6 mice in particular parallel those observed in macrophages from lepromatous and tuberculoid patients, respectively.[22]

Materials and methods

MICE

The mouse strains used in this study were SW and C57Bl/6. Nfs were obtained from 2- to 3-day postnatal mice, and 3- to 4-month-old animals were the source for peritoneal macrophages.

CELL CULTURE PREPARATION

Dissociated Nf cultures

Cultures were essentially obtained by a modified method of Brockes *et al.*[23] Briefly, sciatic and brachial plexus nerves were collected under aseptic conditions, chopped finely and treated with a 1:1 solution of 0·25% trypsin and 0·05% collagenase (Sigma, USA). They were

dissociated by triturating through a 23G needle and plated on culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, USA) and antibiotics, penicillin and streptomycin. For determination of proteins secreted into the culture medium, the cells were plated at a density of 300,000 cell/55 mm Petri dish. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 and maintained for 7–9 days. By the end of this period, Nfs in the culture medium grew Schwann cells. As determined by staining with S-100, a Schwann cell marker, the cultures contained 15% of Schwann cells, indicating 85% of cells to be Nfs. The remaining cells were a mixture of Schwann cells and neuroepithelial cells. Seven- to 9-day-old cultures were used for infection with *M. leprae*.

Peritoneal macrophages

Macrophages were obtained by irrigating the mouse peritoneal cavity with Minimum Essential Medium (MEM). The cells thus obtained were plated on 55 mm Petri dishes (5×10^6 cells/plate) in medium consisting of MEM supplemented with 10% FCS and penicillin. Twenty-four hours later, the cultures were washed free of non-adherent cells and maintained for an additional 5–6 days before infection with *M. leprae* or activation with lipopolysaccharide (LPS).

SOURCE OF *M. LEPRAE* AND INFECTION OF CELL CULTURES

M. leprae was derived from liver and spleen biopsies obtained from experimentally infected armadillos (supplied by Dr E. Storrs, Florida Institute of Technology). The biopsy was aliquoted into small pieces and stored at −70°C until use. To purify *M. leprae* from biopsy, the tissue was homogenized in DMEM until a smooth homogenous suspension containing no observable tissue pieces was obtained. The suspension was centrifuged at 1000 rpm for 10 min to minimize tissue contamination. The supernatant, containing the *M. leprae*, was further subjected to centrifugation at 4000 rpm. The pellet thus obtained was diluted with DMEM, and *M. leprae* counts of solidly stained bacilli were obtained with Zeil Nelson Carbol Fuchsin (ZNCF) stain.[24] Viability of *M. leprae* in the suspension was determined by the FDA-EB staining method[25] and was generally in the range of 80%. The *M. leprae* suspension was stored at 4°C and used within a week, as the viability of *M. leprae* was well maintained within this time span.

Nf and macrophage cultures were infected for 24 h with 10^6 *M. leprae*/ml. As control, Nf cultures fed with heat-killed (autoclaved for 20 min at 120°C at 15 lb pressure) *M. leprae* for 24 h were also included in the study. After 24 h the extracellular bacilli were washed off and the cultures fed with DMEM supplemented with 10% FCS. The bacillary load in Nfs was determined by subjecting cultures on coverslips to ZNCF staining to stain *M. leprae* and 1% methylene blue to stain Nfs. On average, 92% of Nfs were infected after 24 h of infection with *M. leprae* and showed an average bacterial load of 7·5 bacilli/cell.

ACTIVATION OF MACROPHAGES WITH LIPOPOLYSACCHARIDE (LPS)

LPS (2 μg/ml) (Sigma, USA) was added to macrophage cultures for 2 h, after which excess LPS was washed off. Cultures were maintained for a further 24 h in MEM supplemented

with 10% FCS and penicillin following which the conditioned medium was collected and used.

ADDITION OF MACROPHAGE CONDITIONED MEDIUM TO Nf CULTURES

Conditioned medium from uninfected, 3 day post-infected and LPS activated macrophage cultures was centrifuged at 4000 rpm to remove extracellular bacilli and cellular debris. They were added immediately to Nf cultures with an equal volume of DMEM supplemented with 10% FCS and antibiotics and maintained for 72 h.

ESTIMATION OF SECRETORY PROTEINS

Nf cultures in 55 mm petri-dishes were pulsed with ^{14}C-leucine (5-μCi/plate) (Amersham, Denmark) in 2·5 ml leucine-free DMEM supplemented with 10% FCS and antibiotics. ^{14}C-Leucine was added to 3 day, post-*M. leprae* infected, heat-killed *M. leprae* fed HKML and corresponding uninfected Nf cultures. Thereafter, spent medium was collected every 72 h and cultures replaced with fresh medium containing ^{14}C-leucine for a period of 12 days.

Proteins in 2 ml of the collected conditioned medium were precipitated by treating twice with 20% trichloroacetic acid. The protein precipitate was dissolved in 1 N NaOH, added to 10 ml of Bray's fluid and radioactivity determined in a scintillation counter. Part of the culture supernatants was used to determine levels of secreted collagen and fibronectin by ELISA.

ESTIMATION OF LEVELS OF COLLAGEN TYPE I, III AND IV

Conditioned medium (50 μl) from Nf culture was coated at a protein concentration of 200 μg/ml in 96 well Nunc Maxisorb (Denmark) plates at 4°C for 72 h. Similar quantities of DMEM supplemented with 10% FCS was coated as a negative control. The wells were washed off and non-specific blocking carried out with 0·1% BSA in phosphate buffered saline (PBS) at 37°C for 1 h. Then 50 μl of polyclonal goat antibodies to type I, III, and IV (Sera-lab, code 1310, 1330 and 1340, respectively) collagen was added at a dilution of 1:1000 and the mixture incubated for 3 h at 37°C. After washing off excess antibodies with PBS, 50 μl of horseradish peroxidase (HRP) (Dako, Denmark) labelled anti-goat antibodies was added at a dilution of 1:2000 and incubated for 45 min at 37°C. Following extensive washes with PBS, the plates were incubated with 50 μl/well of substrate (4 mg of *o*-phenylinediamine with 4 μl of hydrogen peroxide) for 15 min at 37°C and reaction was stopped by adding 50 μl/well 2·5 N H_2SO_4. The optical density (OD) of the coloured product was read at 492 nm in an ELISA reader.

ESTIMATION OF FIBRONECTIN

Conditioned medium at a protein concentration of 20 μg/ml was coated in 96-well Nunc Maxisorb plates at 4°C for 72 h. DMEM supplemented with 10% FCS at a similar protein concentration was coated as a negative control. Preblocking was done with 0·1% BSA for 1 h at 37°C. Monoclonal antibody to fibronectin (Seralab MAS 224) was used at a dilution of 1:2000 in PBS and incubated at room temperature overnight. After washing off excess antibody, HRP labelled anti-mouse antibody was added at a dilution of 1:1000 and incubated

at 37°C for 45 min. Following extensive washes with PBS, the plates were incubated with 50 μl/well of substrate (4 mg of *o*-phenylinediamine with 4 μl of H_2O_2) for 15 min at 37°C and reaction was stopped by adding 50 μl/well of 2·5 N H_2SO_4. The OD of the coloured product was read at 492 nm in an ELISA reader.

STATISTICAL ANALYSIS

Statistical significance was determined by the Student's unpaired *t*-test. Differences between two variables with significance of $p < 0{\cdot}05$ were considered significant.

Results

RESPONSE OF NfS TO *M. LEPRAE* INFECTION

Total protein secretion

Infection with *M. leprae* or feeding with heat-killed *M. leprae* for 6 days had no effect on secretion of ^{14}C-leucine labelled proteins by Nfs from both SW and C57Bl/6 mice (Table 1). As the age of cultures increased, the amount of radiolabelled proteins secreted by Nfs decreased steadily. However, the trend of protein secretion by infected Nfs versus uninfected Nfs remained unchanged over a infection period of 12 days (Table 1).

Secretion of collagen types I, III, and IV

Nfs from both strains of mice responded with a decrease in collagen secretion on infection with viable *M. leprae* for 6 days. In the SW strain, the decrease was in collagen type IV, and in the C57B1/6 strain, the decrease was observed in the levels of collagen type *I* ($p < 0{\cdot}05$). Secretory levels of the other collagen types were unaffected by infection. Feeding cultures with heat-killed bacilli had no effect on the secretion of any of the collagens studied (Figure 1).

Table 1. Secretion of ^{14}C-leucine labelled proteins by neurofibroblasts. Conditioned medium from uninfected and *M. leprae* infected Nf cultures, pulsed with ^{14}C-leucine in leucine-free DMEM with 10% FCS, was collected every 72 h and radioactive counts of trichloroacetic acid precipitated proteins were determined. Values given are mean cpm of two independent experiments

	SW		C57B1/6	
Days	Uninfected (cpm)	Infected (cpm)	Uninfected (cpm)	Infected (cpm)
6	97,258	102,091	170,184	169,367
9	2,721	2,204	35,311	39,061
12	982	998	1,880	1,610

SECRETION OF FIBRONECTIN

Following infection with *M. leprae* for 6 days, secretion of fibronectin by Nfs was reduced

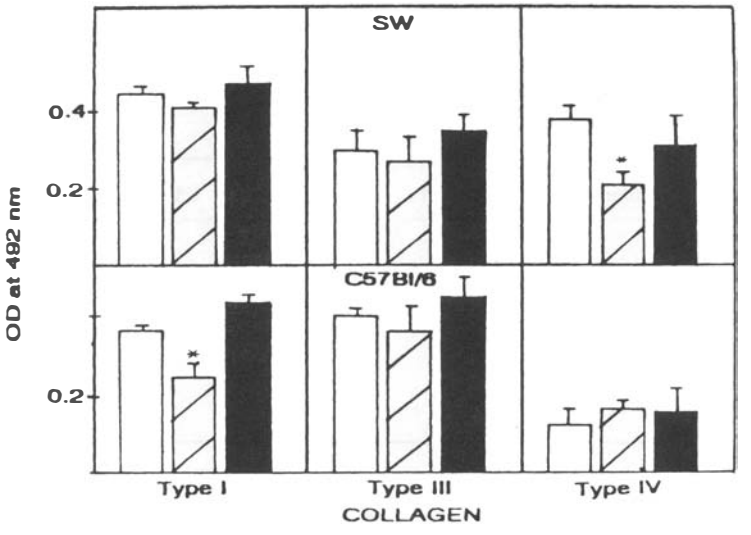

Figure 1. Levels of collagen type I, III and IV secreted by uninfected Nf cultures (□) were compared with Nf cultures that were infected with *M. leprae* (▨) or fed with heat-killed (■) *M. leprae* for 6 days in SW and C57B1/6 strains of mice. Values indicated are mean ± SD of three independent experiments performed in triplicates (*$p < 0{\cdot}05$ as compared to uninfected Nf cultures).

($p < 0{\cdot}01$) in both SW and C57Bl/6 mice. Feeding cultures with heat-killed *M. leprae* had no effect on secretion of fibronectin (Figure 2).

RESPONSE OF NfS TO MACROPHAGE SECRETORY PRODUCTS

Total protein secretion

Nfs from the two strains responded differently to macrophage secretory products. *M. leprae*-infected SW Nfs showed decreased protein secretion on exposure to conditioned medium

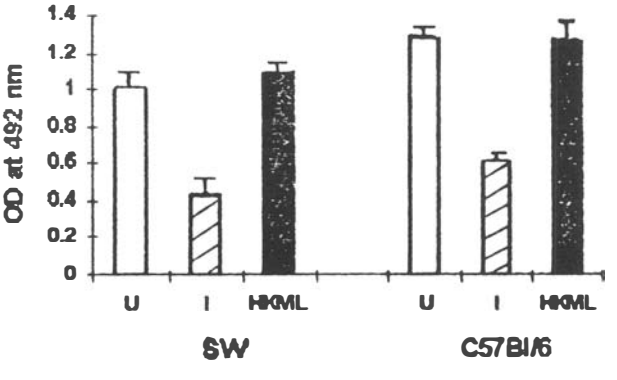

Figure 2. Levels of fibronectin secreted by uninfected Nf cultures (U) were compared with Nf cultures that were infected with *M. leprae* (I) or fed with heat-killed (HKML) *M. leprae* for 6 days. Values indicated are mean ± SD of three independent experiments performed in triplicate.

Table 2. Secretion of ^{14}C-leucine labelled proteins by neurofibroblasts in presence of macrophage conditioned medium. Uninfected (U) or viable (I) *M. leprae* infected Nf cultures were exposed for 72 h to conditioned medium (CM) from uninfected (MoU), 3 day post-infected (MoI) or LPS (MoLPS) activated macrophages cultures. Values are mean cpm of two independent experiments

Nf culture + macrophage CM	SW (cpm)	C57Bl/6 (cpm)
U	98,473	159,374
I	97,901	158,290
U + MoU	106,875	119,761
I + MoU	82,115	119,172
U + MoI	102,090	126,371
I + MoI	63,157	127,695
U + MoLPS	75,369	97,255
I + MoLPS	57,394	112,579

from infected and LPS activated macrophages, while conditioned medium from uninfected macrophages had no effect. In contrast, C57Bl/6 Nfs were refractory to the actions of uninfected, infected and LPS activated macrophage conditioned medium (Table 2).

Secretion of collagens type I, III and IV

Only secretion levels of those collagen types that were altered by *M. leprae* infection were further affected by macrophage secretory products. In SW Nfs, a decrease in collagen type IV was observed in the presence of LPS activated macrophage conditioned medium (Figure 3). In C57Bl/6 Nfs, a generalized decrease was observed in secretion levels of collagen type I on exposure to uninfected, infected or LPS activated macrophage conditioned medium

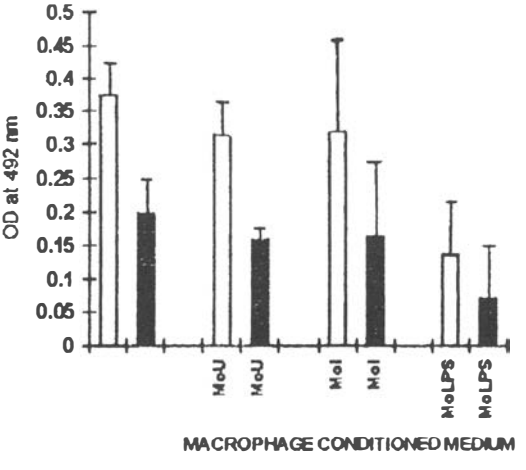

Figure 3. Secretory levels of collagen type IV of uninfected (□) and 3 day post-infected (■) Nf cultures exposed for 72 h to conditioned medium from uninfected (MoU), *M. leprae* infected (MoU) or LPS activated (MoLPS) macrophage culture in SW strain. Values indicated are mean ± SD of three independent experiments performed in triplicate.

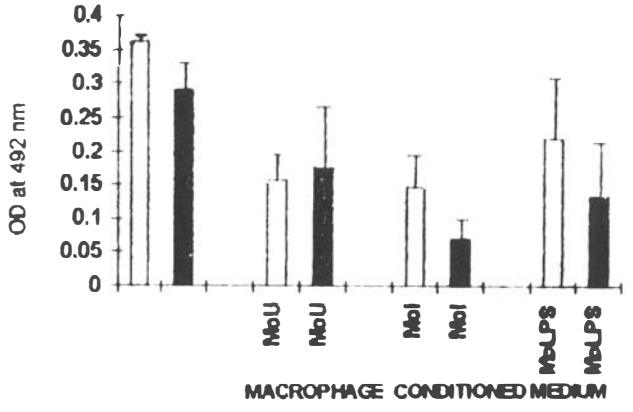

Figure 4. Secretory levels of collagen type I of uninfected (□) and 3 day post-infected (■) Nf cultures exposed for 72 h to conditioned medium from uninfected (MoU), *M. leprae* infected (MoU) or LPS activated (MoLPS) macrophage culture in C57Bl/6. Values indicated are mean ± SD of three independent experiments performed in triplicate.

(Figure 4). Secretory levels of collagen type I and III by SW Nfs and types III and IV by C57Bl/6 Nfs was unaffected in the presence of macrophage conditioned medium (data not given).

Secretion of fibronectin

Following exposure to uninfected, *M. leprae* infected or LPS activated macrophage conditioned

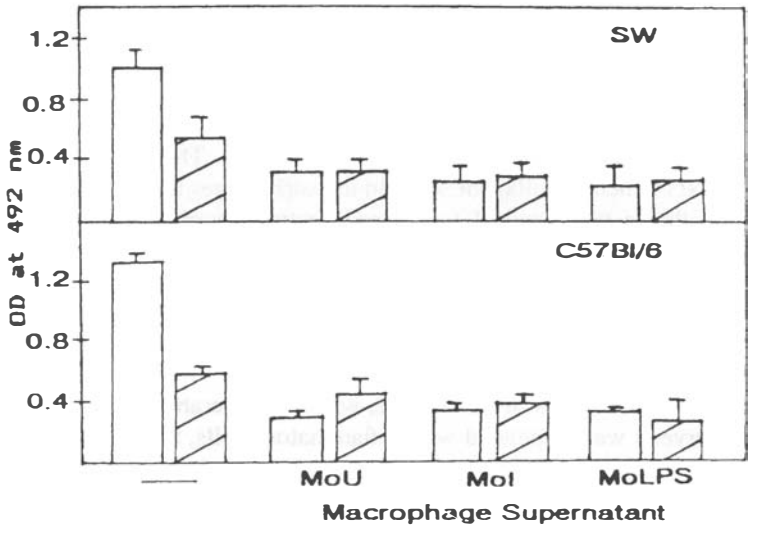

Figure 5. Secretory levels of fibronectin of uninfected (□) and 3 day post-infected ▨) Nf cultures exposed for 72 h to conditioned medium from uninfected (MoU), *M. leprae* infected (MoU) or LPS activated (MoLPS) macrophage culture in SW and C57Bl/6 strains of mice. Values indicated are mean ± SD of three independent experiments performed in triplicate.

medium, the levels of fibronectin secreted by both uninfected and *M. leprae* infected Nfs from the two strains were further decreased (Figure 5).

Discussion

Fibrosis is a normal feature of wound healing. It occurs actively in tissues in response to constant trauma, or replaces areas devoid of cellular contents in a pathological condition. However, recent data implicate fibrosis as the aetiological agent in a number of conditions.[13] In leprosy, the presence of early signs of collagen deposition and later, a general increase in the interstitial collagen,[17] indicate a role for aberrant ECM deposition in the pathogenesis of leprous nerves. Since perturbed Nf activities such as perineurial multilayering, which is an important feature of leprous neuropathy, and increase in cell population are present, the present study was carried out to determine the contribution, if any, of Nfs in fibrosis and thus in the pathogenesis of leprous nerve damage.

Following *M. leprae* infection the secretion of collagens and fibronectin was decreased in Nfs from both the strains. This effect was not observed on feeding cultures with heat-killed bacilli, indicating the necessity of viable bacilli and not just its physical presence for this effect. Similar studies have also shown that alteration in protein secretion level is a feature of viable *M. leprae* infected Schwann cells[18] and macrophages,[22] the other host cells of *M. leprae*.

However, in the present study, alteration in secretion of individual ECM proteins was not reflected by any change in the secretion of total proteins, which remained unaltered on infection with *M. leprae*. Fibroblasts are known actively to secrete other ECM proteins and growth factors. It has been shown that infection with *M. leprae* increases the production of nerve growth factor in mouse Nfs *in vitro*.[20] This discrepancy, therefore, between total protein secretion and secretion of individual ECM proteins observed in the present study might merely indicate a compensatory increase in production of other proteins. This may result in decreased secretion of some ECM proteins not being reflected as a decreased in levels of total secretory proteins.

Though collagenization is a feature of leprous nerves across the spectrum, it is not known which cells or what other factors contribute to the fibrosis. The present study indicates a limited role for Nfs in neural collagenization in the early stages as the secretory levels of the two interstitial collagen, type I and III, following infection was either unchanged or decreased in the two strains. This is in agreement with histological observations of no Nf activity in early leprous nerves. Substantial histological evidence[17] and recent *in vitro*[18] studies strongly indicate that in the early stages collagen deposition may be mainly by Schwann cells. It has already been demonstrated that following *M. leprae* infection, these cells secrete enhanced levels of collagen type I, III and IV.[18]

It is only in the later stages of the disease, when considerable neural damage has taken place and the nerve is well populated with inflammatory cells, that *M. leprae* are observed within Nfs and an increase in their population is seen. As production of the major interstitial collagen, type III, by these cells was unaffected by the actions of macrophage secretory products, this study indicates that neural collagenization in advanced leprous nerves may be of Nf origin. Sustained production of this collagen by Nf, unaffected by macrophage secretory factors, in leprous nerves would lead to collagenization over a period of time. This observation was, however, surprising as macrophages, in addition constitutively to

secreting several fibrogenic enzymes,[26] secrete a repertoire of protein degrading enzymes which includes matrix metalloproteinases that degrade interstitial collagens like collagen type I, stromeolysin that acts on fibronectin and a 72 kDa collagenase that cleaves collagens like type IV.[27–29] In addition, studies have also demonstrated that macrophage derived secretory products themselves induce the production of proteolytic enzymes in responsive cells. The present observations on ECM production in presence of macrophage secretory products reinstates the complex regulatory mechanism that occur for ECM protein metabolism at the site of inflammatory lesions.[30] This is especially signified by the observation of difference in effect of conditioned medium from LPS-treated macrophages on secretion of the different collagen types by Nfs from the two strains. Conditioned medium from LPS activated macrophages would contain high levels of TNF-α which is suppressive in its action on ECM protein metabolism.[31] However, the fibrogenic cytokines present in the conditioned medium may counter this effect, resulting in unaltered levels of some of the collagens that were observed in the presence of conditioned medium in the present study.

The effect of macrophage conditioned medium on secretion of ECM proteins, especially collagens, appears to be in contrast to the response of Schwann cells. It has been shown that macrophage secretory products downregulate the secretion of ECM proteins[18] by Schwann cells. This supports the present observation that Nfs may be the major contributors to neural collagenization only in the later stages of nerve damage in leprosy. However, unlike the important aetiological role of collagen deposition in inflammatory conditions like pulmonary fibrosis and atherosclerosis, collagen accumulation by Nfs in the later stages of nerve damage in leprosy may be just a secondary response to inflammatory damage. The response of Nfs to inflammatory changes may simply be similar to the response of other body tissue fibroblasts to damage, with ECM proteins accumulated by them finally replacing areas devoid of cellular content in damaged leprous nerves.

Acknowledgements

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