Leukaemia inhibitory factor retards the progression of atherosclerosis

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Received 5 September 2002; accepted 26 November 2002

Abstract

Objective: Our previous studies showed that the pleiotropic cytokine leukaemia inhibitory factor (LIF) inhibits the de novo formation of experimental atherosclerotic lesions. The present study examined whether LIF also inhibits progression of pre-existing atheroma.

Methods: Balloon angioplasty was performed on the right carotid arteries of 18 rabbits immediately before placing animals on a cholesterol-enriched diet. After 4 weeks, at which time the intima:media ratio (I:M) was 0.99 ± 0.12 (n = 6), osmotic minipumps containing LIF (n = 6) or saline control (n = 6) were inserted into the peritoneal cavity of each of the remaining rabbits for a further 4 weeks. Arteries were then harvested for analysis. Results: Continuous administration of LIF for the final 4 weeks of an 8-week cholesterol-enriched diet completely inhibited lesion progression in injured carotid arteries (I:M 1.05 ± 0.16) compared with the saline-treated group at 8 weeks (1.62 ± 0.13; P < 0.05). Similarly in contralateral uninjured carotid arteries, LIF treatment prevented an increase in I:M from a baseline of 0.11 ± 0.01 at 4 weeks to 0.15 ± 0.02 at 8 weeks compared with 0.40 ± 0.04 for the saline-treated group at 8 weeks (P < 0.05). LIF reduced the number of macrophages in the neointima of uninjured arteries, but had no effect on the cellular composition of injured arteries. LIF treatment normalised smooth muscle-dependent vasoreactivity to phenylephrine and sodium nitroprusside in both injured and uninjured arteries. Expression and activity of inducible nitric oxide synthase (iNOS) were up-regulated in response to hypercholesterolemia with levels further increased following endothelial denudation. With LIF treatment, iNOS expression was increased in uninjured arteries but marginally reduced in injured arteries. LIF receptors were expressed in both uninjured and injured arteries, with LIF treatment having no significant effect on expression levels.

Conclusion: LIF prevents progression of pre-formed atherosclerotic plaques, affecting lesion size and vascular reactivity. LIF treatment has differential effects within the artery wall, depending on the presence or absence of de-endothelialisation injury.

Keywords: Angioplasty; Atherosclerosis; Cytokines; Smooth muscle; Vasoactive agents

1. Introduction

The cytokine leukaemia inhibitory factor (LIF) is a secreted glycoprotein and member of the interleukin-6 family of cytokines. Originally characterised by virtue of its ability to induce the terminal differentiation and suppress the clonogenicity of myeloid leukaemic cells [1], LIF is now known to possess a diverse range of biological activities in a variety of tissue systems [2]. LIF directs neurotransmitter choice and neuron survival and is under clinical trial as a potential treatment for motor neurone disease [3]. LIF is essential for embryo implantation, and acts directly or synergistically to stimulate osteoblast/osteoclast activity, release of acute-phase proteins by hepatocytes, differentiation of megakaryocytes into platelets, and regulation of angiogenesis. It also promotes proliferation of skeletal myoblasts and some haemopoietic cells, inhibits adipocyte lipoprotein lipase activity and induces cytokine expression in human monocytes. The regulation of these complex processes is mediated by LIF binding to the specific LIF receptor (LIFR)-α subunit which heterodimerises with the common signal transducing subunit for the IL-6 family of cytokine receptors, gp130 [4].

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Previous studies in our laboratory have demonstrated that LIF also inhibits the development of experimental atherosclerosis. Continuous administration of LIF to cholesterol-fed rabbits reduced plasma cholesterol through up-regulation of liver low density lipoprotein receptors, with aortic fatty streak formation almost entirely inhibited [5–7]. Neointima formation induced by either a silastic cuff or de-endothelialisation injury (i.e. in the presence or absence of an intact endothelium) was also significantly inhibited [8]. Further investigation showed that cuff-induced neointimal thickening was augmented by treatment with 1-NAME (an inhibitor of nitric oxide synthase, NOS), suggesting that the inhibitory effect of LIF may, at least partly, be due to induction of NOS activity in the artery wall.

While these studies showed that LIF inhibits the de novo formation of a neointimal thickening in vessels with no prior atheroma, the more important question clinically is whether LIF can inhibit the progression, or induce regression, of established atherosclerotic lesions. The present study therefore utilised a rabbit model of atherosclerosis to determine the effect of LIF on pre-existing atheroma. This model combines arterial injury with hypercholesterolemia and produces an advanced lesion with a central core of lipid-filled macrophages covered by a fibrous cap of proliferated smooth muscle cells (SMC), similar to the human lesion [9]. We report here that LIF prevents the rabbits and lengths of proliferated smooth muscle cells (SMC), similar to the (injured right and unmanipulated left) were removed from lipid-filled macrophages covered by a fibrous cap of immediately prior to LIF treatment.

Also at 4 weeks, an Alzet osmotic minipump (Model 2ML4) containing saline (group II) or hLIF (group III) was inserted into the peritoneal cavity of each of the 12 remaining rabbits (6/group). The pumps deliver 2.5 μl/h, with a mean volume of 2.167 ml, and therefore are potentially active for 36 days. The dose of LIF was 30 μg/kg per day, which from our earlier studies is known to be effective in lowering plasma cholesterol and inhibiting the development of fatty lesions [6]. At the same time, the cholesterol in the Chow of these 12 rabbits was reduced to 0.5% as continued consumption of 1% cholesterol can result in liver failure after 3–6 months. This dietary regime consistently results in elevation of plasma cholesterol from normal levels of 1.0±0.01 to 10.8±0.6 mM after a 4-week diet, with LIF treatment reducing plasma cholesterol to 7.6±0.5 mM. After 4 weeks of LIF (or saline) infusion, groups II and III were sacrificed with an overdose of sodium pentobarbitone.

Immediately following sacrifice, the carotid arteries (injured right and unmanipulated left) were removed from rabbits and lengths of ~1.5 cm used fresh to determine their reactivity to contractile agonists. The remaining (~2.0 cm) lengths of artery were used for histology, immunochemistry and NOS assay. For histology and immunochemistry, artery segments were fixed in 4% paraformaldehyde, and then each piece cut into four 5.0-mm segments and embedded in paraffin. Several sections (5 μm) were cut at intervals of 500 μm, yielding six sampling sites per artery. For NOS assays, tissue was flash frozen in liquid nitrogen and stored at ~80 °C.

The investigation was approved by the University of Queensland Animal Experimentation Ethics Committee and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 88-23, revised 1996).

2.4. Carotid artery reactivity (organ bath studies)

Segments of carotid artery were trimmed of adventitial connective tissue and cut into 3-mm rings. Three rings from each carotid artery were suspended on fine stainless steel wire hooks to a tension of 30 mN in a jacketed organ bath (25 ml) containing Tyrode’s solution maintained at 35 °C and continuously gassed with 95% O₂:5% CO₂. After a 1-h equilibration, the rings were contracted with phenylephrine (Sigma, St. Louis, MO; 10⁻⁶ M) to determine contractile function. Following rinse-out of the solution, cumulative (0.5 log unit) concentration–response (contraction) curves were determined for either 5-hydroxytryptamine (Sigma) or phenylephrine. Concentration–re-

2. Methods

2.1. LIF

Recombinant human LIF (hLIF) was a gift from the Australian Medical Research and Development (AMRAD) Corporation (Victoria, Australia).

2.2. Animals

A total of 24 male 18-week-old New Zealand White rabbits weighing ~2.5 kg were obtained from commercial colonies. They were caged individually, maintained on a 12-h day/night cycle, and fed ad libitum with standard rabbit chow (with or without 1% cholesterol) and small quantities of fruit and vegetables.

2.3. Experimental protocol

At time 0, six rabbits were placed on normal rabbit chow (untreated controls). The remaining 18 rabbits were subjected to balloon catheter denudation injury of the right carotid artery as previously described [8] and immediately placed on a 1% cholesterol diet. At 4 weeks, by which time lesions with a central core of lipid-laden foam cells and a fibrous cap had developed [9], six rabbits (group I) were sacrificed. This group provided an indication of lesion size immediately prior to LIF treatment.
response (relaxation) curves following contraction with 5-hydroxytryptamine (3 × 10^{-7} M) were determined for acetylcholine and sodium nitroprusside (both from Sigma). Finally, after washout, the contractile response \(E_{\text{max}}\) and EC50 values (i.e. the concentration of drug that elicited 50% of the maximum response) were calculated and data compared between treatment groups.

2.5. Histology/morphometric analysis

For morphometric analysis, paraffin sections were stained with toluidine blue. Images were captured via an Olympus microscope with a solid state colour video camera linked to a computer and the colour images stored and analyzed using the Mocha image analysis system (Jandel Scientific, CA, USA). Arteries were circumscribed by digital planimetry and the areas within the lumen, internal elastic lamina (IEL) and external elastic lamina (EEL) measured directly. The intimal area (encompassing the atherosclerotic plaque) was calculated by subtracting the lumen from the IEL area. The size of the plaque was expressed as the area of lesion/(intima as a ratio of the total wall area enclosed by the EEL (i:M, intima:media).

2.6. Immunohistochemistry

Paraffin sections were dewaxed and brought to water before pre-incubation in 0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.2 (phosphate-buffered saline, PBS) containing 1% bovine serum albumin (BSA; Sigma) to block non-specific binding. They were then incubated for 2 h at room temperature with primary antibodies to smooth muscle actin (HHF35), macrophages (RAM 11) or proliferating cell nuclear antigen (PCNA; PC10) (all mouse monoclonal antibodies; Dako, Carpinteria, CA). Antibody binding was visualised using biotinylated secondary antibody (anti-mouse Ig; Silenus, Melbourne, Australia), followed by streptavidin-horse radish peroxidase (Silenus) as third layer and 3,3-diaminobenzadine (DAB; Sigma) as chromagen; sections were counterstained with haematoxylin. The total number of neointimal cells and the number of macrophages in a full cross-section of artery were determined by visual counting. Three to six cross-sections were analysed per artery and the percentage of macrophages calculated. The proliferation index was calculated by the formula:

\[
\frac{\text{PCNA positive staining cells}}{\text{haematoxylin staining cells}} \times 100\%
\]

2.7. Nitric oxide synthase assay

iNOS activity in the cytosolic extracts of carotid arteries was measured by the conversion of \(^{[14]}\text{H}\)arginine to \(^{[14]}\text{H}\)citrulline using a modified version of the method used by Busse and Mulsch [10]. Frozen tissue segments were pulverized in a mortar-and-pestle and then disrupted in ice-cold homogenization buffer (25 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA) by repeated passages through a 25-gauge needle. The homogenate was centrifuged at 13 000 rpm and the resulting supernatant snap-frozen in small aliquots and stored at −20 °C until required. Protein was estimated by Nano-Orange Protein Assay Kit (Molecular Probes, Eugene, OR). Equal amounts of artery cytosol were added to 40 μl of reaction buffer (25 mM Tris–HCl pH 7.4, 3 μM tetrahydropterin (Sigma), 1 μM flavin adenine dinucleotide (Sigma), 1 μM flavin adenine mononucleotide (Sigma), 1 mM NADPH (Sigma) and 0.8 μCi \(^{[14]}\text{H}\)arginine (Amersham, UK)) and incubated at 37 °C for 1 h. The assay was terminated by the addition of ‘stop buffer’ (50 mM Tris–HCl pH 5.5, 5 mM EDTA). Reacted samples were applied to equilibrated Dowex AG50X-8 resin and transferred to spin cups that were centrifuged at 13 000 rpm for 30 s. Following addition of scintillant (ReadySafe, Beckman Instruments, CA), eluted \(^{[14]}\text{H}\)citrulline was quantified in a Beckman LS 6000TA counter.

2.8. Western blot analysis

Protein extracts of rabbit carotid arteries were prepared by homogenization in ice-cold lysis buffer (10 mM Heps, 50 mM NaCl, 0.5 mM sucrose, 1 mM EDTA, 0.05 mM EGTA, 0.05% β-mercaptoethanol, 0.5% Triton X-100, 0.6 mM spermidine) supplemented with a protease inhibitor cocktail (Sigma) to a final concentration of 0.3% (v/v). Samples were further disrupted by repeated passages through a 25-gauge needle and centrifuged at 13 000 rpm for 10 min. The supernatant was collected and stored at −20 °C until required. Equal amounts of total protein (60 μg), as estimated by Nano-Orange fluorescence, were separated by electrophoresis under denaturing conditions on an 8% SDS–polyacrylamide gel [11]. Parallel gels were stained with Coomassie blue to verify the loading of equal amounts of protein. Separated proteins were electroblotted onto PVDF membrane (Millipore, Bedford, MA) [12]. After drying, membranes were blocked for 1 h with blocking buffer (5% non-fat dried milk powder, 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) before incubation with monoclonal antibodies to iNOS (Transduction Laboratories, Lexington, KY; diluted 1:500), LIFR-α or gp130 (Santa Cruz, Biotechnology, Santa Cruz, CA; diluted 1:200) followed by an alkaline phosphatase conjugated anti-mouse immunoglobulin secondary antibody (Promega, Madison, WI; diluted 1:2000). Immunoreactive bands were visualised with BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt)/NBT (nitro blue tetrazolium chloride) (Boehringer Mannheim, Germany). Membranes were scanned by densitometry and semi-quantitation of immunoreactive bands performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).
2.9. Statistical analysis

Statistical analyses were performed using the statistical software package ‘SIGMA STAT’ (Jandel Scientific, CA, USA). I:M ratios were subjected to log transformation and analysed by one-way ANOVA (Student-Newman-Keuls comparison). For results expressed as percentages, data were subjected to arcsin square root transformation [13] before analysis. Cell proliferation indices and reactivity to contracting and relaxing agents were compared by one-way ANOVA followed by the Tukey-Kramer test. Results are expressed as mean±S.E.M. In all statistical analyses, a P value of <0.05 was considered significant.

3. Results

3.1. Lesion development

In order to determine the effect of LIF treatment on progression of pre-existing atheroma, two different experimental lesions were induced in the carotid arteries of rabbits: a simple fatty streak was developed in the left carotid artery in response to a 1% cholesterol diet, and a more complex atherosclerotic lesion was induced in the contralateral right carotid artery by a combination of diet and denudation injury. After 4 weeks, rabbits in group I were sacrificed in order to establish lesion size immediately prior to commencement of LIF administration. At the elastic lamina, the majority of the lesion was comprised of a more complex atherosclerotic lesion (Fig. 2b). LIF treatment (group III) for the final 4 weeks of cholesterol diet. were sometimes a small number of macrophages in the media, possibly as the result of damage to the internal elastic lamina.

After 4 weeks cholesterol diet (group I), fatty streak development in uninjured arteries was relatively modest with I:M = 0.11±0.01 (Fig. 1). Macrophages were prominent in these lesions (Fig. 2a) with 12.1±4.1 macrophages per cross-section (Fig. 3a). After 8 weeks (group II) there was a significant increase in lesion size (I:M = 0.59±0.04; P<0.05) in the saline-treated animals, due to both the continued accumulation of macrophages (significantly increased to 114.9±23.2 macrophages per cross-section; P<0.05) (Figs. 2b and 3a) as well as a marked increase in SMC (Fig. 2b'). LIF treatment (group III) retarded lesion progression with I:M = 0.15±0.02 (not significantly different from that at 4 weeks; Fig. 1). Both total cell number (129.4±29.2 per section) and the number of macrophages (61.4±15.5 per section) within lesions was significantly less than that in the saline-treated group at 8 weeks (233.3±45.4; P<0.05), although the proportion of macrophages (42.8±3.8%) was unaltered (Figs. 2c, c' and 3a). Thus in these lesions, LIF treatment appears to have a similar effect on both macrophages and SMC, reducing the involvement of both cell types proportionally. Interestingly, proliferation indices were similar for all three experimental groups (9.8±5.7, 10.7±3.9 and 8.1±2.6% for groups I, II and III, respectively; Fig. 3b).

The neointima of injured arteries was much larger than uninjured contralateral arteries after 4 weeks cholesterol diet (group I; I:M = 0.99±0.12) (Fig. 1). SMC were the predominant cell type in these lesions (Fig. 2d'), but there was also considerable macrophage involvement (Fig. 2d). There were sometimes a small number of macrophages in the media, possibly as the result of damage to the internal elastic lamina. The majority of α-smooth muscle actin positive cells were close to the internal elastic lamina, indicating their recent migration from the media, but there were also a small number of cells near the lumen. After 8 weeks, lesion size for group II animals (I:M = 1.62±0.13) was significantly higher than that at 4 weeks (P<0.05). However there was no increase in the total number of cells within lesions, and the proportion of macrophages was not significantly different (Fig. 3a), nor was the proliferation index significantly altered (Fig. 3b). Macrophages were seen throughout the neointima (Fig. 2e), as were SMC that formed randomly-oriented concentric layers. A layer of α-smooth muscle actin-positive cells formed an overlying fibrous cap (Fig. 2e'). LIF treatment (group III) prevented an increase in lesion size such that the I:M (1.05±0.16) was not significantly different from that of arteries at 4 weeks (group I). LIF treatment had no effect on cell proliferation (22.1±6.2; Fig. 3b) or cellular composition, with the proportion of macrophages the same as that in group II (48.4±2.9 vs. 47.3±2.8%; Fig. 3a). However, cell density was higher and there were differences in the distribution of cells within the neointima. The majority of macrophages were located close to the lumen of the artery (Fig. 2f), while α-smooth muscle actin positive cells were scattered throughout the intima and their orientation was more uniformly circumferential (Fig. 2f').
Fig. 2. Immunohistochemical staining of uninjured (left) carotid arteries (a–c) and injured (right) carotid arteries (d–f) with antibodies specific for macrophages (RAM 11; a–f) and smooth muscle actin (HHF35; a’–f’) after 4 weeks’ cholesterol diet (group I; a, a’; d, d’); 8 weeks’ cholesterol diet with saline treatment for the final 4 weeks (group II; b, b’; e, e’) and 8 weeks’ cholesterol diet with LIF treatment for the final 4 weeks (group III; c, c’; f, f’). ↑, internal elastic lamina. Scale bar represents 10 μm.
mN; \( P < 0.05 \) compared with values for arteries of control and group II animals, but not significantly different from the 4-week value (Fig. 4b').

A cholesterol-enriched diet significantly decreased the maximal vasoconstrictor responses to the \( \alpha \)-adrenoceptor agonist, phenylephrine, in uninjured carotid arteries at 4 and 8 weeks (\( E_{\text{max}} \) 42.0±4.7 and 50.0±2.0 mN, respectively) and injured arteries after 8 weeks (\( E_{\text{max}} \) 42.6±4.9 mN) compared with the control group (\( E_{\text{max}} \) 67.6±4.5 mN; \( P < 0.05 \)) (Fig. 4c–c'). LIF treatment normalised the response of injured arteries (\( E_{\text{max}} \) 69.7±7.5 mN), a value not significantly different from the control group, but significantly increased compared with the saline-treated group II at 8 weeks (\( E_{\text{max}} \) 42.6±4.9 mN; \( P < 0.05 \); Fig. 4c'). Constrictions mediated by 5-hydroxytryptamine were unaltered in cholesterol-fed rabbits, with or without LIF treatment (data not shown).

3.3. iNOS expression

Western analysis showed that expression of iNOS protein (as evidenced by a single 130-kDa immunoreactive signal) was 3-fold higher in injured arteries at 4 weeks than in uninjured arteries at the same time-point (not shown). After 8 weeks (group II), iNOS expression in injured arteries was 6-fold higher than in their uninjured counterparts (\( P < 0.05 \); Fig. 5a). With LIF treatment, iNOS protein expression in uninjured arteries was 33-fold greater than in the saline-treated group II (\( P < 0.05 \)). However LIF-treatment of injured arteries had no significant effect on iNOS protein levels.

3.4. LIF receptor expression

Examination of expression of LIF receptor subunits by Western analysis showed expression of LIFR-\( \alpha \) and gp130 in both injured and uninjured arteries of cholesterol-fed rabbits (Fig. 6). LIF treatment had no significant effect on the expression of receptor subunits.

4. Discussion

Our previous studies showed that continuous infusion of LIF inhibits the formation of fatty streaks and neointimal thickenings/atherosclerotic lesions in a rabbit model [5,6,8]. However, for LIF to be an effective treatment of human disease, it must be able to inhibit the progression of pre-existing atherosclerotic plaque. Importantly, the pres-
ent study demonstrates that LIF treatment does indeed prevent the progression of pre-existing atherosclerotic lesions (both fatty streaks and more advanced lesions), affecting lesion size and vascular reactivity. It also shows for the first time that receptors for LIF are present in the artery wall and are up-regulated after injury. The results suggest that the effect of LIF is partly mediated by activation of iNOS which is also up-regulated by injury and further modulated by LIF administration.

LIF treatment of pre-existing lesions for the final 4 weeks of an 8-week cholesterol-enriched diet inhibited the increase in lesion size, with I:M not significantly different from those observed at 4 weeks but significantly less than the saline-treated group at 8 weeks. LIF reduced the total number of cells within ‘fatty streak’ lesions. In injured arteries, however, lesion progression (measured by increased I:M) was not associated with an increase in cell number. These results are in accordance with previous studies in our laboratory and others which show that after the initial proliferative response to arterial injury, cell numbers within the neointima do not increase but cell density decreases and lesion development is primarily due to the synthesis and accumulation of matrix proteins such as collagen [14–16] (Thomas, unpublished data). Clowes and co-workers [17] further showed that in regions lacking endothelium, intimal SMC proliferation persisted for up to 12 weeks after injury but, as in the present study, the total cell number within lesions did not change. This suggests the development of an equilibrium between cell proliferation and apoptosis [18]. With LIF treatment, cells within the lesions were more ordered and cell density was higher, suggesting that LIF may be regulating processes involved in matrix remodelling. LIF has been shown to play a role in regulating matrix synthesis and degradation in a number of different cell types. In cardiac fibroblasts LIF reduces collagen synthesis and metalloprotease activity [19] while in osteoclasts it stimulates metalloprotease MMP-13 activity and the metalloprotease inhibitor, tissue inhibitor of metalloproteases-1 (TIMP-1) [20]. LIF has also been reported to influence proteoglycan metabolism in chondrocytes [21].

In contrast to our earlier studies in which administration of LIF from the time of injury almost completely inhibited the development of a smooth muscle-rich neointima [5], LIF treatment of pre-existing atheroma had no effect on cell proliferation. This dichotomy may be explained by our recent in vitro studies which suggest that the timing of LIF administration determines the cellular response [22]. LIF signalling in vascular cells occurs via the JAK-STAT, ERK MAPK and p38 MAPK pathways, which regulate the expression of downstream genes that are thought to contribute to the inhibition of VSMC proliferation and neointimal formation. The ability of VSMC to respond to LIF is abrogated when cells are pre-exposed to mitogens
such as interleukin-1β which are released at the time of vascular injury (unpublished data). The inhibition of LIF signalling via a ‘cross-talk’ mechanism suggests that for LIF to be effective as an anti-proliferative agent, treatment must commence before cells are exposed to endogenous mitogens. In the present study where LIF treatment did not commence until after injury (and thus after exposure to mitogen), SMC proliferation was not affected. Clowes and Clowes [23] have shown that SMC in the media commit to entering the cell cycle within the first 24 h after injury and if this process is inhibited they do not subsequently proliferate; the result is a reduced SMC mass within the neointima and a corresponding reduction in lesion size. The efficacy of LIF may thus be enhanced by timing administration to inhibit this initial SMC response.

The impairment of vascular reactivity in response to hypercholesterolemia and mechanical injury is well documented [24–26] with atherosclerotic lesions exhibiting increased sensitivity to vasoconstrictors [27] and impaired endothelium-dependent relaxation [28]. In the present study, LIF restored smooth muscle reactivity, normalising the impaired responses to the endothelium-independent agonists phenylephrine and sodium nitroprusside. This suggests that LIF may be affecting a common pathway of contraction, or may exert an effect on multiple pathways that contribute to smooth muscle contraction. However LIF treatment had no apparent effect on endothelial function such as interleukin-1β which are released at the time of vascular injury (unpublished data). The inhibition of LIF signalling via a ‘cross-talk’ mechanism suggests that for LIF to be effective as an anti-proliferative agent, treatment must commence before cells are exposed to endogenous mitogens. In the present study where LIF treatment did not commence until after injury (and thus after exposure to mitogen), SMC proliferation was not affected. Clowes and Clowes [23] have shown that SMC in the media commit to entering the cell cycle within the first 24 h after injury and if this process is inhibited they do not subsequently proliferate; the result is a reduced SMC mass within the neointima and a corresponding reduction in lesion size. The efficacy of LIF may thus be enhanced by timing administration to inhibit this initial SMC response.

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differential effects on iNOS, augmenting iNOS expression and activity in uninjured arteries, while at the same time reducing expression in injured arteries where iNOS expression and activity are already elevated. Thus by regulating iNOS activity, LIF treatment may maintain NO at beneficial levels while preventing excessive production of NO which has been linked to the production of harmful oxidative products such as peroxynitrite [33].

In summary, LIF is an effective anti-atherosclerotic agent, retarding the progression of both pre-existing fatty streaks and more complex atherosclerotic lesions. Our results also show that LIF has differential effects within the artery wall, depending on the presence or absence of de-endothelialisation injury and suggest that the timing of LIF administration is critical in determining its effects.

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia, the Heart Foundation of Australia and AMRAD Corporation. The authors wish to acknowledge the assistance of Nicole Smith, Andrew Fenning and Vincent Chan with vascular reactivity studies.

References