Role of interleukin-1β in the pathogenesis of diabetic retinopathy

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Aim: To examine the role of cytokine interleukin-1β (IL-1β) in retinal capillary cell death in diabetes.

Methods: The effect of glucose on the expression of IL-1β was measured in the bovine retinal endothelial cells. The role of IL-1β in the accelerated endothelial cell loss was determined by investigating the effect of human recombinant IL-1β on their apoptosis in normal and high glucose conditions, and was confirmed using interleukin-1 receptor antagonist (IL-1ra).

Results: High glucose increased IL-1β expression by 60% compared with cells incubated in 5 mM glucose (p<0.05). Incubation of cells with IL-1β increased NO levels by about 80% and activated NF-kB by 40%. In the same cells apoptosis was increased by 70% and caspase-3 activity was increased by 40%. Supplementation of IL-1β in 20 mM glucose medium further increased nitric oxide and NF-kB, and accelerated apoptosis, and addition of IL-1ra significantly decreased glucose induced abnormalities and apoptosis.

Conclusions: IL-1β accelerates apoptosis of retinal capillary cells via activation of NF-kB, and the process is exacerbated in high glucose conditions. These studies suggest a possible role of IL-1β in the development of retinopathy in diabetes, and offer a possible rationale to test IL-1β receptor antagonists to inhibit the development of diabetic retinopathy.

METHODS

Capillary cells
Endothelial cells were prepared from bovine retina as described earlier.14–17 The cells were grown to 80% confluence in petri dishes coated with 0.1% gelatin in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal calf serum (heat inactivated), 10% Nu serum, heparin (50 μg/ml), endothelial growth supplement (25 μg/ml), and antibiotic/antimycotic in an environment of 95% O₂ and 5% CO₂.

Confluent cells from fourth to seventh passage were split and incubated under normal glucose (5 mM glucose) or high glucose (20 mM glucose) conditions for 1–10 days in the presence or absence of human recombinant IL-1β (0–10 ng/ml, R&D Systems, Minneapolis, MN, USA). Control incubations containing 20 mM mannitol were run simultaneously to rule out the effect of increased osmolarity. Each experiment was repeated with at least three separate cell preparations.

Western blot
The cells were homogenised in 30 mM Tris-HCl buffer containing 10 mM EGTA, 5 mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 15 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 1 mM NaN₃VO₄. The homogenate was

Abbreviations: IL, interleukin; iNOS, inducible nitric oxide synthase; NO, nitric oxide.
centrifuged at 5000 × g for 5 minutes at 4°C to remove cell debris. Protein (40 μg) was separated on 8–15% denaturing polyacrylamide gel, and then transferred to nitrocellulose membranes. The membranes were blocked in the wash buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% Tween 20) containing 5% milk, followed by the incubation with a polyclonal antibody against IL-1β (R & D Systems) at 1:500 dilutions. The membranes were washed three times (10 minutes each), and incubated for 1 hour at room temperature with horseradish peroxidase linked antirabbit IgG (1:2500 dilution; Amersham Biosciences, Amersham, UK), and developed using ECL-Plus western blotting detection kit (Amersham Biosciences). Kaleidoscope prestained molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) were run simultaneously on each gel. To ensure equal loading among the lanes the expression of housekeeping protein, β-actin or GAPDH, was determined. After blocking the membrane for the desired proteins, the membranes were incubated with stripping buffer (62.5 mM Tris-HCl pH 6.8, 100 mM mercaptoethanol, 2% sodium dodecyl sulfate) at 50°C for 30 minutes, washed and incubated with mouse monoclonal antibody against β-actin (Sigma Chemicals, St Louis, MO, USA) or GAPDH (Biodesign International, Saco, ME, USA). IgG horseradish peroxidase conjugated antimouse antibody was used as the secondary antibody, and the membranes were developed using ECL-Plus western blotting detection kit. Each sample was analysed in duplicate.16 17

Nitrite production
Nitrite production was measured using Greiss reagent.18 19 The absorbance was measured at 540 nm, and the nitrite concentration was calculated from a sodium nitrite standard curve.

NF-κB
NF-κB activation was determined by performing western blots to measure the increased expression of the p65 subunit of NF-κB. Protein was separated on 10% denaturing polyacrylamide gel, blotted onto nitrocellulose membrane, and after blocking the membrane was incubated with the antibody against NF-κB (p65; 1:500 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA).

Cell death
Cell death was determined by ELISA using Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Indianapolis, IN, USA), and confirmed by measuring the enzyme activity of caspase-3.

Nitrite production and cell death
The relative amounts of mono- and oligonucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones respectively as described by us previously.17 The cytoplasmic fraction of the cells was transferred onto a streptavidin coated microtiter plate and incubated for 2 hours at room temperature with a mixture of peroxidase conjugated anti-DNA and biotin labelled antihistone. The plate was washed thoroughly, incubated with 2,2’-Azino-di-[3-ethylbenz-thiazoline sulfonate] diaminomium salt (ABTS, Roche Diagnostics), and absorbance was measured at 405 nm. After separation of the cytoplasmic fraction, the nuclear pellet was suspended in 50 mM sodium phosphate buffer (pH 7.5) containing 2 mM NaCl, 0.05 mM Na2HPO4 (pH 7.5) and sonicated. DNA was measured in this fraction, and apoptosis was normalised to the amount of DNA.16 17

Caspase-3
Activation of caspase-3 in the retina was determined by measuring the cleavage of fluoregenic substrate specific for caspase-3, N-acetyl-Asp-Glu-1-Val-asp-7-amino-4 trifluoromethyl coumarin (DEVD-AFC, Biomol Research Laboratories). The fluorescence signal emitted by cleavage of the substrate was quantified at excitation and emission wavelengths of 400 nm and 505 nm respectively as routinely used in our laboratory.16 17 Each sample was measured in duplicate.

Statistical analysis
Data are reported as mean (standard deviation), and experimental groups were compared using the non-parametric Kruskal-Wallis test followed by Mann-Whitney test for multiple group comparison. Similar conclusions were reached also by using ANOVA with Fisher or Tukey.

RESULTS

Effect of high glucose on IL-1β expression in retinal endothelial cells
Incubation of retinal endothelial cells in 20 mM glucose for 3–5 days resulted in about 60% increase in the protein expression of IL-1β compared with the cells incubated in 5 mM glucose (p<0.05, fig 1). When the incubation with 20 mM glucose was extended to 10 days the expression of IL-1β did not show any additional increase (data not shown). Addition of 20 mM mannitol, instead of glucose, failed to increase IL-1β expression, suggesting that the glucose induced increase in IL-1β was not due to an increase in the osmolarity. Despite the differences in the expression of IL-1β, the content of the intrinsic protein, β-actin or GAPDH did not vary among various lanes. This shows that high glucose results in increased cytokines levels in the retinal endothelial cells.

Effect of IL-1β on nitric oxide and cell death
The data in figure 2A show that the incubation of endothelial cells in 5 mM glucose medium containing 10 ng/ml IL-1β for 3 days resulted in the increase of nitric oxide (NO) by over 80% compared with that from the cells incubated without IL-1β. In the same cells addition of IL-1β resulted in the activation of NF-κB, as shown by 40% increase in the expression of 65kD subunit of NF-κB (fig 3).
compared with 5 mM glucose, and were also incubated with 50 ng/ml IL-1 or 100 ng/ml IL-1. (A) NO content was measured in the endothelial cells incubated in 20 mM glucose containing four different concentrations of recombinant IL-1. (B) To confirm the effect of IL-1 on glucose induced NO, the cells were also incubated with 50 ng/ml IL-1ab or 100 ng/ml IL-1ra for three days. Each measurement was performed in duplicate. *p<0.05 compared with 5 mM glucose, and **p<0.05 compared with 20 mM glucose.

IL-1β also increased the apoptosis of endothelial cells by 70%, compared with the cells incubated in 5 mM glucose without IL-1β (fig 4A). To further confirm that IL-1β has an important role in the accelerated endothelial cell apoptosis, the effect of IL-1β was determined on the activity of the enzyme important for the execution of apoptosis, caspase-3. As shown in figure 4B, addition of 10 ng/ml IL-1β in 5 mM glucose medium resulted in 40% increase in the activity of caspase-3.

Effect of IL-1β on high glucose induced NO and cell death

As reported by us previously, incubation of retinal endothelial cells in 20 mM glucose medium for three days increased NO levels by 2.5 fold compared with the cells incubated in 5 mM glucose alone. However, when 20 mM glucose medium was supplemented with 0–10 ng/ml IL-1β, there was a dose dependent increase in NO levels, and the values obtained with 10 ng/ml IL-1β were almost twofold higher compared with those obtained from 20 mM glucose alone (fig 2; the concentration of IL-1β used in the subsequent experiments was 10 ng/ml). Similarly, IL-1β also increased glucose induced activation of NF-xB by additional 50% (fig 3).

We have shown that high glucose accelerates the apoptosis of retinal endothelial cells, and the results are presented in figure 4 confirming that the incubation of endothelial cells in 20 mM glucose medium for 5 days notably increased their apoptosis by over 90% compared with that observed from the cells incubated in 5 mM glucose medium. However, addition of IL-1β during the incubation with 20 mM glucose further increased the apoptosis by 2.5 fold (fig 4A), and activated caspase-3 by additional 30% compared with the cells incubated in 20 mM glucose medium alone (fig 4B).

Neutralisation of IL-1β and high glucose induced NO and cell death

In order to confirm that IL-1β plays a crucial role in glucose induced accelerated endothelial cell apoptosis, the effect of neutralisation of IL-1β by IL-1ab (50 ng/ml monoclonal antibody), or IL-1ra (100 ng/ml, R & D Systems) was determined on the NO levels and cell apoptosis. As shown in figures 2B–4, addition of IL-1ra significantly decreased glucose induced increases in NO levels and NF-xB expression, and decreased the apoptosis of the retinal endothelial cells. Further, co-incubation of 10 ng/ml IL-1β with either IL-1ab or IL-1ra in 20 mM glucose medium also prevented glucose or IL-1β induced abnormalities and apoptosis in the endothelial cells. The beneficial effects of IL-1ab were observed even when the concentration of IL-1β was reduced to 0.5 ng/ml (data not shown).

DISCUSSION

This is the first report showing increased levels of inflammatory cytokine, IL-1β, in the retinal capillary cells in high glucose conditions, and the effect of IL-1β on their accelerated apoptosis. Our results also show that an
IL-1β can induce the expression of many genes whose promoters are regulated through complex interactions with NF-κB. NF-κB, heterodimer with two subunits, p50 and p65, is regulated by redox control mechanisms, and transcriptionally activates various cellular genes involved in immune response, inflammation, and oxidative stress. It is localised in the subretinal membranes and in the microvessels, and is usually stored in its inactive form in the cytosol. Following IL-1β stimulation, it is translocated into the nucleus where it initiates the transcription of many genes involved in apoptosis. We have shown that NF-κB is activated in the retina in diabetes and in the capillary cells of the retina in high glucose conditions. Our studies have suggested that the activation of NF-κB is an early event in the development of retinopathy, that is sustained when the retinal capillary cell death is accelerating and histopathology is developing. Further, the activation of NF-κB seems to be associated with the failure of retinopathy to arrest after re-establishment of normal glycaemia (Knowluru RA, et al. Unpublished data). Romeo et al have shown that NF-κB activation in retinal pericytes plays a significant role in the hyperglycaemia induced accelerated loss of pericytes observed in diabetic retinopathy. Data are provided here showing that IL-1β activates NF-κB in the retinal endothelial cells that is amplified in high glucose conditions, and suggest that in diabetes IL-1β might play an important role in the activation of NF-κB and its signalling pathways.

In retina IL-1β gene expression is present in glial cells and endothelial cells, and the expression is significantly upregulated in the glial cells, endothelial cells, and neutrophils recruited into the retina during reperfusion after induced retinal ischaemia. Administration of IL-1β into the vitreous of Lewis rats is reported to cause retinal inflammatory response that is accompanied by the breakdown of the vascular blood retinal barrier. This induces the recruitment of leukocytes that enter the retina predominantly through the retinal vasculature, and migrate through retinal endothelial cells, suggesting that IL-1β is an important factor in the pathogenesis of human retinal inflammation. IL-1β leads to the induction of vascular endothelial growth factor in vascular smooth muscle cells. Similar abnormalities are observed in the retina in diabetes; the capillaries in the retina become non-perfused, obliterated and ischaemic. VEGF levels are increased, and the number of platelet fibrin thrombi increases. These proinflammatory changes even leukostasis are some of the earliest changes observed in the retina of diabetic animals, and administration of aspirin, an anti-inflammatory compound, inhibits the development of retinopathy in diabetic dogs, suggesting that this inflammatory cytokine might play an important role in the pathogenesis of diabetic retinopathy.

Retinal capillary cells are shown to undergo accelerated cell death in the pathogenesis of diabetic retinopathy, and apoptosis has been implicated as one of the mechanisms in their accelerated death. Apoptotic cells themselves can become procoagulant, and that can create a proinflammatory environment. The levels of proapoptotic protein Bax are higher in the capillary cells of the retina. We have shown that the activity of caspase-3 is increased in the rat retina at duration of diabetes when capillary cell death and histopathology can be detected. IL-1β has been shown to induce apoptosis in retinal neuronal cells during reperfusion ischaemia, and this supports our results showing that IL-1β exerts proapoptotic effects on retinal endothelial cells that are aggravated in high glucose conditions.

IL-1β is considered as one of the most potent stimuli for inducible form of nitric oxide synthase (iNOS), and it stimulates NO production by a dual pathway—that is, by the transcriptional induction of iNOS protein and by the
Interleukin-1 and diabetic retinopathy


Tannous M, Veluthakal R, Amin R, et al. Long exposure to high glucose enhances the IL-1β and high glucose are, perhaps, acting together during the progression of diabetic retinopathy. Our studies offer a possible rationale to test IL-1β receptor antagonists (already being tested for inflammatory and immune disorders) to inhibit the development of diabetic retinopathy.

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