Growth factor protection against cytokine-induced apoptosis in neonatal rat islets of Langerhans: role of Fas

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Abstract Treatment of neonatal rat islets of Langerhans with combined cytokines (interleukin-1β 10^{-10} M, tumour necrosis factor-α 10^{-10} M, interferon-γ 5 U/ml) led to extensive cell death, which was potentiated by Fas activation with the anti-Fas cytolytic antibody JO2. Pre-treatment with insulin (25 ng/ml) or insulin-like growth factor-1 (10^{-8} M) gave only partial protection against cell killing, but prevented the Fas-mediated component. In the absence of cytokine treatment, Fas-mediated killing was not observed.
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Key words: Growth factor; Cytokine; Fas; Apoptosis; Islet of Langerhans

1. Introduction

Cytokines inhibit insulin secretion in rat islets largely through the formation of nitric oxide via inducible nitric oxide synthase [1]. Cytokines, including interleukin-1β (IL-1β), are also able to induce cell death (necrotic and apoptotic), in rat [2] and human pancreatic islets [3,4] and insulin containing cell lines [2,5]. We have previously shown that insulin-like growth factor-1 (IGF-1) protects rat islets from cytokine-mediated apoptosis, partly through decreasing nitric oxide synthase (NOS) expression [6]. It has recently been reported that IGF-2 protects rat islets from cytotoxic and apoptotic effects of cytokines [7]. Insulin administered in vivo protects non-obese diabetic (NOD) mice from insulitis and diabetes [8] and may protect islets from cytokine inhibition or cell death but in vitro protective effects have not previously been demonstrated.

There is evidence that the selective destruction of human [9] and NOD mouse [10] pancreatic β-cells may be the result of apoptosis, although this is not seen in all cases (V. Hadjivassiliou and I. Green, submitted for publication). One of the mediators of apoptosis believed to be involved in β-cell apoptosis is Fas (Apo-1, CD95), a 45 kDa transmembrane cell surface receptor protein and a member of the tumour necrosis factor (TNF) receptor family [11]. The interaction of a functional Fas receptor with its ligand, FasL, or with an agonistic antibody results in apoptosis ([12] and see review [13]). There is growing evidence of a role for Fas-mediated apoptosis in the destruction of β-cells seen in the development of insulin-dependent diabetes [14]. Fas expression can be induced on pancreatic β-cells in human [9] and mouse islets of Langerhans [11,15] through priming with the cytokine, IL-1β, but it has not previously been reported on rat β-cells.

The aims of this study were (1) to determine whether growth factors (IGF-1, insulin) would protect neonatal rat islets from apoptosis induced by the combined cytokines IL-1β, TNFα and interferon-γ (IFN-γ), (2) to see if combined cytokine treatment induces Fas expression on rat islets and (3) to determine if growth factor protection against cytokine-induced apoptosis occurs via interference with the Fas-mediated apoptotic pathway.

2. Materials and methods

Reagents were obtained from the following sources: tissue culture RPMI 1640 and foetal calf serum from Gibco BRL (Paisley, UK); anti-Fas antibody, JO2 (PharMingen, San Diego, CA, USA). The cytokines used were human recombinant IL-1β (a gift from Glaxo Wellcome, Stevenage, UK), human recombinant TNF-α and IGF-1 (gifts from the National Institute for Biological Standards and Control, Potters Bar, UK) and rat recombinant IFN-γ (Genzyme, West Malling, Kent, UK).

2.1. Islet isolation and culture
Islets of Langerhans were isolated under aseptic conditions from collagenase-digested pancreata of 7–12 day old Sprague-Dawley rats or Lewis rats [16]. Batches of 150–500 islets were cultured in RPMI 1640 medium containing 5.5 mM glucose, penicillin (50 U/ml), streptomycin (50 µg/ml) and 5% foetal calf serum for 2 days prior to growth factor pre-treatment and cytokine treatment.

2.2. Treatment of islets with growth factors and cytokines
Groups of up to 300 islets were treated in complete RPMI culture medium in 60 mm Petri dishes and cultured for 48 h at 37°C in an atmosphere of 5% CO_2:95% air before analysis. Growth factors used were IGF-1 (10^{-8} M) and insulin (25 ng/ml). Islets were treated with growth factors for 24 h. Where growth factor pre-treatment occurred, prior to cytokine treatment, the same dish was used and cytokines were added in fresh medium to the islets for a further 48 h unless otherwise stated.

Isolated islets were incubated at 37°C with the human recombinant cytokines IL-1β (10^{-10} M) and TNF-α (10^{-10} M) and rat recombinant IFN-γ (5 U/ml), except in Fig. 1 where concentrations were IL-1β and TNF-α (5×10^{-11} M), IFN-γ (2 U/ml).

2.3. Fas activation
Pancreatic islets, 200 from each treatment group, were trypsinised into single cells and then split into two equal fractions. One fraction had no further additions and the other had the cytolytic anti-Fas antibody JO2 added at a 1:20 dilution of the antibody as supplied. All samples were then incubated at 37°C and cell numbers determined at timed intervals, by counting on a haemocytometer slide.

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2.4. Use of acridine orange cytochemistry to visualise apoptotic cells

Acridine orange cytochemistry was used for determination of apoptotic nuclei and has been validated by us, alongside other measurements of apoptosis [2]. Modifications of our published acridine method were used. Following treatments, islets were trypsinised, centrifuged at $200 \times g$ and single cells resuspended in approximately 20 μl medium. A 10 μl aliquot was mixed with an equal volume of 20 μg/ml acridine orange, transferred to a microscope slide and viewed using a broad band fluorescence filter set, excitation range 450–490 nm. Under these conditions, in control cells acridine orange, a weak fluorescent base, remains sequestered in acidic compartments in the cytoplasm where its fluorescence is quenched, the cytoplasm is orange and the nucleus a dull green-black. Cells which are apoptotic have bright green condensed nuclei usually in rounded fragments; post-apoptotic cells typically have green, rather than orange cytoplasm, with apoptotic nuclei. Nuclei were counted and the percentages of apoptotic and post-apoptotic cells calculated.

2.5. Statistical analysis

Results are presented as means ± S.E.M. Probability was determined by multivariate analysis of variance using the statistical package GLIM (NAG, Oxford, UK), one-way analysis of variance, or Student's *t*-test as indicated in the legends to figures.

3. Results

3.1. Growth factors

Cell death by apoptosis was increased by combined cytokine treatment and decreased when islets were pre-treated with insulin or IGF-1 (Fig. 1). Neonatal rat islet cell rates of apoptosis were not significantly altered by growth factor exposure alone (Fig. 1).

Fas activation was observed following incubation of cytokine-treated islet cells with a cytolytic anti-Fas antibody (Fig. 2). At early time points – 15, 30 even 60 min – of incubation with antibody, cell numbers decreased very significantly versus cells incubated with no antibody addition (Fig. 2). Fas expression on cells was increased by combined cytokine treatment from $2.3 \pm 0.1\%$ ($n = 12$) to 9.3 ± 0.15 ($n = 6$) as measured by flow cytometry on a Coulter Epics Profile 11 analyser (Coulter Electronics, Krefeld, Germany), using the same anti-Fas antibody at a low dilution (1:250 of the antibody as supplied), together with a fluorescence-conjugated anti-hamster second antibody.

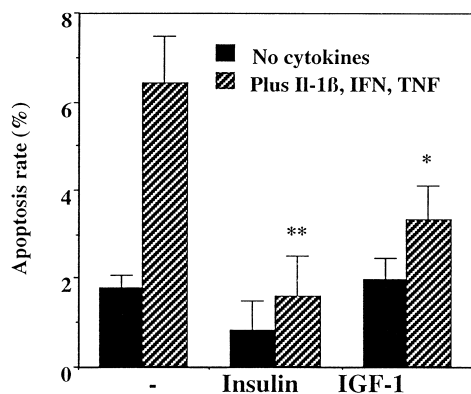When equal numbers of control islets and 48 h cytokine-

Fig. 1. Combined cytokine treatment of neonatal rat islets (IL-1β, TNF-α, IFN-γ, 48 h) results in increased apoptosis as determined by acridine orange cytochemistry. Pre-treatment (24 h) of neonatal rat islets with growth factors insulin (25 ng/ml) or IGF-1 (10^{-8} M) significantly lowered cytokine-induced apoptosis. Results are the means ± S.E.M. of apoptosis determinations in at least 1500 nuclei in five separate experiments. *$P < 0.05$, **$P < 0.01$ versus cytokine-treated without growth factor pre-treatment, by Student's *t*-test.
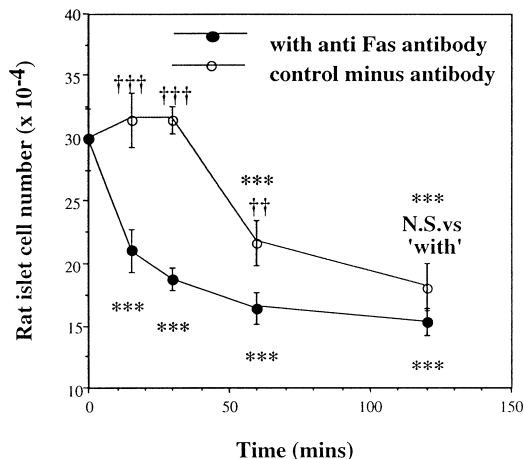

Fig. 2. Neonatal rat islets were treated with IL-1β, TNF-α, IFN-γ for 48 h. Following trypsinisation, cells were split into two groups, one was treated with anti-Fas antibody and the other had no additions. Both groups were incubated at 37°C, aliquots were removed for cell counting at timed intervals. Addition of the anti-Fas antibody resulted in rapid onset of cell death. Results are the means ± S.E.M. of 15 replicates within three experiments. ***$P < 0.001$ versus value at time 0; ††$P < 0.01$, †††$P < 0.001$ versus treatment with anti-Fas antibody, by one-way ANOVA.

treated islets were made into single cells, there was a dramatic difference in cell loss between the two groups (cells/ml: control 99×10^4; cytokine 29.3×10^4). At the start of the experiment (time 0 min) it was clear that cytokine-treated islets, pretreated with growth factors, showed improved cell recovery on trypsinisation (cells/ml: cytokine+insulin 42.5×10^4; cytokine+IGF-1 37.6×10^4) (Fig. 3). However, this was only partial protection, as there was still a significant reduction in cell survival versus non-cytokine-treated controls.

The contribution of Fas activation to growth factor modulation of cytokine-induced cell death is shown in Fig. 3. Although pre-treatment with either growth factor gave only limited protection following trypsinisation, both growth factors prevented the Fas-mediated component of killing (Fig. 3). In the absence of cytokine treatment, no Fas-mediated killing was observed (data not shown). When a multivariate analysis of variance was performed on the data, using the statistical package GLIM, the following conclusions were apparent: (i) there was no significant difference in potency between insulin and IGF-1; (ii) the anti-fas antibody caused a highly significant decrease in survival in cytokine-treated cells ($P < 0.001$), although the size of the effect varied between experiments; (iii) insulin and IGF-1 gave a highly significant ($P < 0.001$) increase in survival in cytokine-treated cells; (iv) the protective effect of insulin and IGF-1 was largely confined to anti-Fas-treated cells. In the absence of anti-Fas antibody, the protective effect was significantly smaller ($P < 0.001$).

4. Discussion

We have shown that IGF-1 and insulin protect neonatal rat pancreatic islets from cytokine-mediated cell death and protect islet cells from Fas-mediated apoptosis. The protection to single cells that growth factor pre-treatment offers is most apparent in the first 2 h following trypsinisation. After this time, cell loss reaches a plateau and differences in cell loss
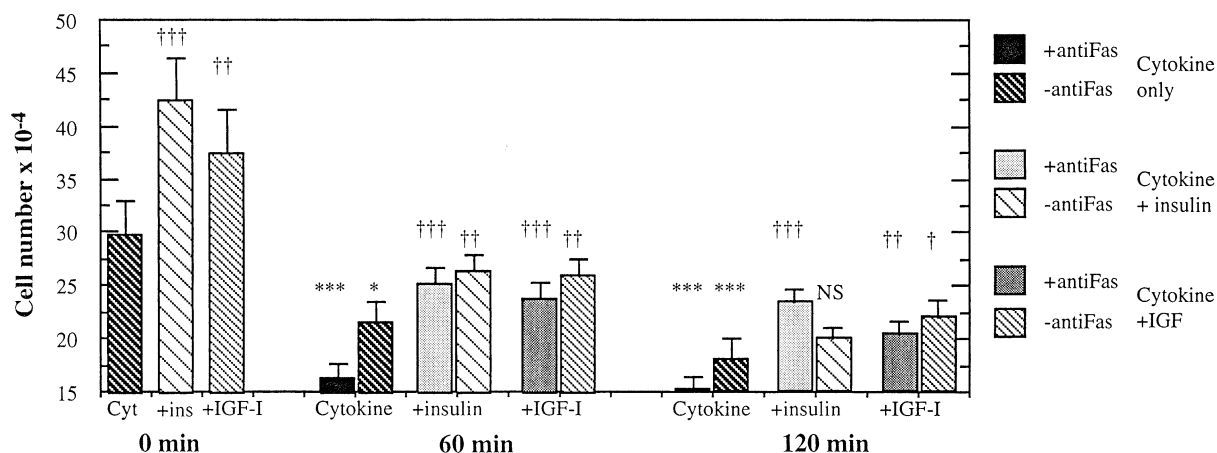
Fig. 3. Neonatal rat islets were pre-treated with insulin or IGF-1 for 24 h followed by 48 h treatment with combined cytokines (IL-1β, TNF-α, IFN-γ). After trypsinisation (time 0), single cells were incubated at 37°C with or without cytolytic anti-Fas antibody. Growth factors significantly protected islet cells from Fas-mediated cell death. At 60 min, insulin maintained viability of $4.6 \pm 0.7 \times 10^4$ extra cells. In the presence of accelerated killing by Fas antibody insulin was more protective and this number almost doubled ($8.9 \pm 0.2 \times 10^4$, $P < 0.005$). Insulin was effective at 120 min, IGF-1 protected at 60 but not 120 min. $^{††}P < 0.02$, $^{†††}P \leq 0.000$ versus treatment without growth factor, $*P < 0.05$, $***P < 0.001$ versus number of cytokine-treated cells at the start of the experiment. Multivariate analysis of variance conclusions are in Section 3.

between growth factor-pre-treated and non-pre-treated cells become non-significant (data for up to 5 h not shown).

When growth factor-pre-treated, cytokine-treated islets are trypsinised and incubated with anti-Fas antibody the growth factors appear to allow cell loss at a level similar to that of the cytokine-treated islets not incubated with the anti-Fas antibody. Growth factor protection offered to the non-antibody-treated islet cells, post trypsinisation, is barely significant compared to improvement in cell survival in islet cells exposed to the anti-Fas antibody. It would appear that the growth factors indeed protect the single cells from Fas-mediated apoptosis. There are two main mechanisms by which protection could be conferred. The first is through a physical protection, i.e. growth factor pre-treatment resulting in an accumulation of extracellular matrix around islet cells which may prevent the Fas receptor from being bound by the anti-Fas antibody. Support for this possibility comes from work using another growth factor, transforming growth factor β (TGF-β). TGF-β induction of extracellular matrix proteins has been shown to make murine fibroblasts resistant to tumour necrosis factor cytotoxicity [17].

The second mechanism of protection could be occurring through modulation of signalling pathway(s) involved in apoptosis. IGF-1 has previously been shown by us to reverse cytokine-mediated apoptosis and to decrease NOS expression. Some of its anti-apoptotic effects may be due to lowering NOS expression as an arginine analogue also confers protection [6]. Furthermore, if Fas expression is NO-dependent to a degree, it is possible that IGF-1 may provide partial protection from Fas-mediated apoptosis via this route.

It is more likely that there are multiple signalling pathways for both IGF-1 and insulin protection from apoptosis. Growth factors, including IGF-1 and insulin, have been shown to trigger the activation of phosphatidylinositol 3'-kinase (PI3K) [18]. PI3K signalling is important to cell survival and its activation can result in suppression of apoptosis [19], including in cells which have become detached from their extracellular matrix [20]. IGF-1 has been purported to inhibit apoptosis in other cell types via the PI3K and mitogen-acti-

vated protein kinase (MAP kinase) pathways [21]. There may well be a role for this signalling pathway in suppression of Fas-mediated apoptosis.

The cytokines IFN-γ, TNF-α and IL-1β are all capable of inducing Fas expression on neonatal rat islets. The level of Fas expression is increased when they are used in combination and increases further with increased IFN-γ dosage [22]. We have shown that Fas-expressing islet cells incubated with the agonistic anti-Fas antibody experience a significantly greater cell loss when compared to those not exposed to the antibody. This indicates that islets, when primed to express Fas through cytokine treatment, are capable of undergoing Fas-mediated apoptosis for example in the presence of the Fas ligand.

That cytokines are damaging to pancreatic islets has been known for a number of years and the link with Fas expression has been shown in other species. It is not known whether endogenous Fas ligand contributes to islet cell killing or has contributed prior to trypsinisation. Results of timed cell counts show that the cross-linking of the Fas receptor results in rapid onset of apoptosis, but it is not possible to say whether this is in addition to, or in place of, further late apoptosis, as cell survival is limited once islets have been made into single cells. It is also interesting to note that in islet cells where β-cell surface antigen expression revealed 90% β-cells, these same cells showed modest expression of Fas ($\ll 20\%$) [22], which raises questions about the timing and regulating of Fas induction by cytokines.

Although Fas-mediated cell death may play a role initially following trypsinisation and Fas activation, it is clearly not the only mechanism by which cytokine-treated cell die. However, it is the first time that functional Fas activity has been shown in neonatal rat β-cells and, more importantly, that the growth factors insulin and IGF-1 directly affect the Fas-mediated apoptotic pathway.

References

[1] Southern, C., Schulster, D. and Green, I.C. (1990) FEBS Lett. 276, 42–44.

[2] Di Matteo, M.A., Loweth, A.C., Thomas, S., Mabley, J.G., Morgan, N.G., Thorpe, J.R. and Green, I.C. (1997) Apoptosis 2, 164–177.

[3] Loweth, A.C., Williams, G.T., James, R.F.L., Scarpello, J.H.B. and Morgan, N.G. (1988) Diabetes 47, 727–732.

[4] Rabinovitch, A., Sumoski, W., Rajotte, R.V. and Warnock, G. (1990) J. Clin. Endocrinol. Metab. 71, 152–156.

[5] Ankarcrona, M., Dypbukt, J., Brüne, B. and nicotera, P. (1994) Exp. Cell Res. 213, 172–177.

[6] Mabley, J.G., Belin, V.D., John, N.E. and Green, I.C. (1997) FEBS Lett. 417, 235–238.

[7] Petrik, J., Arany, E., McDonald, T.J. and Hill, D.J. (1998) Endocrinology 139, 2994–3004.

[8] Atkinson, M.A., Maclaren, N.K. and Luchetta, R. (1990) Diabetes 39, 933–937.

[9] Stassi, G., Todaro, M., Richiusa, P., Giordano, M., Mattina, A., Sbriglia, M.S., Lo Monte, A., Buscemi, G., Galluzzo, A. and Giordano, C. (1995) Transplant. Proc. 27, 3271–3275.

[10] O'Brien, B.A., Harmon, B.V., Cameron, D.P. and Allan, D.J. (1997) Diabetes 46, 750–757.

[11] Yamada, K., Takane-Gyotoku, N., Yuan, X., Ichikawa, F., Inada, C. and Nonaka, K. (1996) Diabetologia 39, 1306–1312.

[12] Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T. and Nagata, S. (1993) Nature 364, 806–809.

[13] Hale, A.J., Smith, C.A., Sutherland, L.C., Stoneman, V.E.A., Longthorne, V.L., Culhane, A.C. and Williams, G.T. (1996) Eur. J. Biochem. 236, 1–26.

[14] Chervonsky, A.V., Wang, Y., Wong, F.S., Visintin, I., Flavell, R.A., Janeway Jr., C.A. and Matis, L.A. (1997) Cell 89, 17–24.

[15] Signore, A., Annovazzi, A., Procaccini, E., Beales, P.E., Spencer, J., Testi, R. and Ruberti, G. (1997) Diabetologia 40, 1476–1479.

[16] Dunger, A.M., Cunningham, J., Delaney, C.A., Lowe, J.E., Green, M.H.L., Bone, A.J. and Green, I.C. (1996) Diabetes 45, 183–189.

[17] Chang, N.-S. (1995) J. Biol. Chem. 270, 7765–7772.

[18] Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B.A. (1996) EMBO J. 15, 6541–6551.

[19] Kauffman-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. and Evan, G. (1997) Nature 385, 544–548.

[20] Khwaja, A., Rodriguez-Viciana, P., Wennström, S., Warne, P.H. and Downward, J. (1997) EMBO J. 16, 2783–2793.

[21] Párrizas, M. and LeRoith, D. (1997) Endocrinology 138, 1355–1358.

[22] Harrison, M.J., Dunger, A., Berg, S. and Green, I.C. (1998) Diabet. Med. 15, S22 abstract 18.