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Endothelial cells as cytotoxic effector cells: cytokine-activated rat islet endothelial cells lyse syngeneic islet cells via nitric oxide

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Summary In vivo, each beta cell is located in proximity to at least one capillary islet endothelial cell. Rat aorta and islet endothelial cells can be activated in vitro to express inducible nitric oxide synthase by a cytokine mixture of tumour necrosis factor-α, gamma-interferon, and interleukin-1β and to produce high concentrations of nitric oxide. We have performed co-culture experiments with rat islet endothelial cells together with isolated syngeneic islet cells at low target : effector ratios with or without previous cytokine challenge of endothelial cultures. Co-cultures were always free of exogenous cytokines, which were removed prior to addition of islet cells. We found that pre-activated, in contrast to resident islet endothelial cells, at a target : effector ratio as low as 1 : 1 almost completely lysed syngeneic beta and non-beta cells within 24 h of co-culture. Lysis by pre-activated islet endothelial cells was found to be preceded by DNA damage found in 46 % of islet cells after 8 h of co-culture with pre-activated vs 7 % with resting islet endothelial cells. Lysis was blocked to control levels in the presence of the nitric oxide synthase inhibitor N^G-methyl-L-arginine. With the results presented here, we demonstrate for the first time, that activated endothelial lining cells can express effector cell activity and thus can contribute to local tissue destruction, especially in organs that are densely capillarized such as pancreatic islets. [Diabetologia (1997) 40: 150–155]

Keywords Cytotoxicity, diabetes mellitus, endothelia, nitric oxide.

Endothelial cells (EC) have long been considered to be only a mechanical barrier of blood vessels. However, evidence is accumulating for a decisive role of EC in immune responses [1]. Upon exposure to local signals such as cytokines, EC undergo profound alterations in gene expression and cell function. Thus, activated EC participate in leukocyte recruitment by producing chemoattractants, by regulated expression of adhesion molecules and also by modulating local blood flow. In addition, endothelial expression of MHC class II molecules as well as production of cytokines (interleukins: IL-1, IL-6, IL-8) [1], oxygen radicals [2, 3] and/or high concentrations of nitric oxide (NO) [4–6] may actively participate in local inflammatory reactions. Specific NO synthase inhibitors partially suppress or delay insulin-dependent diabetes mellitus (IDDM) in animal models [7–10] suggesting that in vivo NO contributes to the development of IDDM presumably released by activated macrophages infiltrating the pancreatic islets of Langerhans early during development of IDDM in animal models [11–13]. Indeed, in vitro experiments showed lysis of syngeneic islet cells mediated by activated macrophages via NO [14]. However, constitutive cell populations of islets also bear the potential for inducible nitric oxide synthase (iNOS) expression and high output NO synthesis, namely the capillary islet endothelial

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Abbreviations: IEC, Islet endothelial cells; iNOS, inducible nitric oxide synthase; NMA, N^G-methyl-L-arginine; ECGS, endothelial cell growth supplement; TNF-α, tumour necrosis factor alpha; PBS, phosphate buffered saline; NO, nitric oxide

cell (IEC), as has been shown previously [6]. In vivo each insulin-producing beta cell is located in close proximity to at least one capillary IEC, which thus could potentially contribute to destructive processes. We established a co-culture system of pure rat IEC with syngeneic isolated islet cells and showed for the first time, that the capillary-lining cells are capable of becoming immune effector cells in an inflammatory surrounding and thus must be considered as possible contributors to tissue breakdown preceding autoimmune diabetes.

Materials and methods

Materials. N^G-methyl-L-arginine acetate salt (NMA), sulphanilamide, naphtylethylendiamine, collagenase from *Clostridium histolyticum* and EC growth supplement (ECGS) were purchased from Sigma (Deisenhofen, Germany), recombinant human tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) from Pharma Biotechnologie Hannover (Hannover, Germany) and recombinant rat gamma-interferon (gamma-IFN) from HyCult Biotechnology (Leiden, The Netherlands). All cells were cultured in RPMI 1640 (pH 7.2) without nitrate containing 4 mmol/l glucose and supplemented with 6×10^4 U/l penicillin, 60 mg/l streptomycin, 1 mmol/l sodium pyruvate, 2 mmol/l glutamine, 10 mmol/l non-essential amino acids × 100, 23.8 mmol/l $NaHCO_3$, 10 mmol/l HEPES (all from Gibco, Heidelberg, Germany) and 10 % heat inactivated fetal calf serum (FCS) (Boehringer Mannheim, Mannheim, Germany).

Animals. Male Wistar rats (weight 200 g) from the University breeding facility received a standard diet ("Sniff-R"; Sniff, Soest, Germany) and tap water ad libitum.

Isolation of cells. IEC were isolated by outgrowth from isolated rat pancreatic islets as described [6]. Briefly, pancreatic islets were harvested by ductual injection of collagenase, submitted to gradient centrifugation and hand picked. Freshly isolated whole islets were cultured in RPMI 1640/10 % FCS supplemented with 100 μg ECGS/ml for 6–8 days, respectively, depending on the degree of endothelial cell outgrowth. Islets were then removed, IEC detached by trypsin treatment, placed onto plastic culture dishes in RPMI/FCS and subcultured. Characterization of IEC was performed using specific endothelial markers as described [6]. Removal from culture dishes for each passage was performed by treatment with 0.05 % trypsin/0.02 % EDTA in isotonic NaCl for about 3 min. After the second passage, the cells were frozen in RPMI/FCS/10 % DMSO at – 80 °C until use. Islet cells were obtained from isolated islets after dissociation into single-cell suspensions exactly as described previously [15]. Subsequently, the islet cells were purified from non-endocrine cells by an adherence step in culture dishes for 18 h.

Co-culture of endothelial and islet cells and quantification of cell lysis. Only IEC of passage 4–6 were used for the co-culture experiments. Cells were cultured in 48 multiwell tissue culture plates (Becton Dickinson, Lincoln Park, NY USA) to a density of about 9×10^4 cells/well and activated with a mixture of 500 U/ml IL-1β, 300 U/ml TNF-α and 100 U/ml gamma-IFN. After a challenge for 24 h, the IEC were washed three times with fresh medium to remove the cytokines. Isolated islet cells, purified by overnight adherence, were then added at various amounts to achieve the appropriate target : effector cell ratios. At the end of the co-culture, cell lysis was determined by trypan blue exclusion. For each well, 2×10^2 islet cells or more were counted. Islet cells could easily be distinguished from the elongated and tightly adherent endothelial cells. For electron microscopy preparations IEC were cultured in permanox eight-well tissue culture chambers (Nunc, Naperville, IL USA) under conditions exactly as above. To avoid loss of cells due to transfer steps during fixation, dehydration and embedding, all steps were performed in the permanox chambers. Briefly, the cells were fixed with 2 % glutaraldehyde in 0.1 mol/l cacodylate-HCl buffer for 15 min and postfixed with 1.5 % OsO_4 for 40 min at 4 °C. Specimens were dehydrated in a graded ethanol series and embedded in a thin layer of Spurr's epoxy resin. After polymerization, the epoxy layer containing the cells was removed from the plastic support and embedded in a manner oriented to allow relocation of cells in ultrathin sections. Specimens were analysed in a coded fashion. From each preparation more than 2×10^2 islet cells were analysed by transmission electron microscopy from 4–5 ultrathin sections cut from different planes of the cell layers. Cell viability was judged from electron density of cytoplasm together with preservation of intact structures of cellular organelles and continuity of cell membrane. Beta and non-beta cells were distinguished by secretory granule morphology.

Determination of DNA strand breaks. For recognition of DNA strand breaks at single cell level, the method of in situ nick-translation was performed as described [16]. Briefly, co-cultures were dried on the slides and fixed in acetone. Endogenous peroxidase activity was inhibited by incubation with 0.3 % H_2O_2 in methanol for 30 min. The nick-translation reaction was performed for 15 min at room temperature in 50 mmol/l Tris-HCl, pH 7.5, containing 3 μmol/l biotin-dUTP, 5 U/100 μl Kornberg polymerase, 3 μmol/l each of dGTP, dCTP, and dATP and 0.1 mmol/l dithiothreitol (all components from Boehringer Mannheim). The slides were then washed three times in phosphate buffered saline (PBS) and processed for immunocytochemical detection of biotin. Unspecific antibody binding was blocked by preincubation with PBS containing 0.1 % thimerosal and 10 % FCS for 10 min. The incorporated biotin-dUTP was visualized by the peroxidase reaction (Vectastain kit; Camon, Wiesbaden, Germany) using diaminobenzidine as substrate. Stained cells were determined by counting more than 3×10^2 islet cells in at least two different wells.

Determination of nitrite concentrations. Nitrite concentrations were determined by diazotization reaction performed in Pro-Bind assay plates (Becton Dickinson). Culture supernatants (100 μl) were mixed with 50 μl 1 % (w/v) sulphanilamide dissolved in 2.5 % H_3PO_4. After 5 min, 50 μl 0.3 % (w/v) naphtylethylendiamine dissolved in 2.5 % H_3PO_4 was added. After adding 20 μl methanol to remove air bubbles, the absorbance at 540 nm was measured in a microplate reader using culture medium incubated without cells as a blank and $NaNO_2$ as a standard.

Statistical analysis

Data are given as arithmetical means ± SD. *p*-values were calculated using analysis with Student's *t*-test (two-tailed for independent samples).

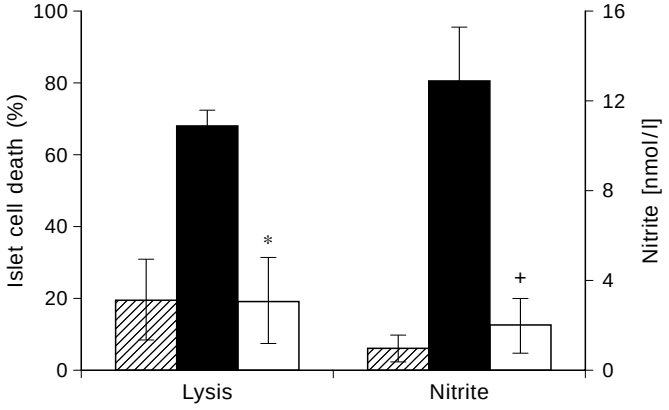

Fig. 1. Lysis of islet cells by islet endothelial cells (IEC). Isolated islet cells were co-cultured with resident (▨) or preactivated IEC at a target : effector cell ratio of 1 : 3 in the absence (■) or presence (□) of 0.5 mmol/l NMA. After 24 h, islet cell lysis was determined by trypan blue exclusion and nitrite concentrations were determined in the culture supernatants. Values are means ± SD of 3–4 individual experiments. *p*-values compare pre-activated IEC with pre-activated IEC + NMA. * $p < 0.007$; + $p < 0.0001$

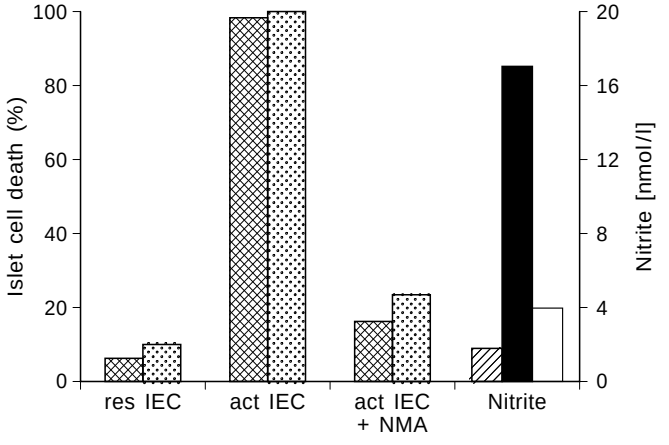

Fig. 2. Lysis of beta and non-beta cells by IEC as determined by electron microscopy. Isolated islet cells were co-cultured with resident (res) or pre-activated (act) IEC at a target : effector cell ratio of 1 : 1 in the absence or presence of 0.5 mmol/l NMA. After 24 h, cultures were processed for electron microscopy. For each specimen 2×10^2 or more islet cells were examined (beta cells, ▩; non-beta cells, □). Nitrite concentrations in the coculture supernatants with resident (▨) or pre-activated IEC in the absence (■) or presence (□) of 0.5 mmol/l NMA were determined. Results of two experiments are shown

Results

Rat IEC layers were pre-activated by the addition of cytokines (500 U/ml IL-1β, 300 U/ml TNF-α and 100 U/ml gamma-IFN) which were removed by extensive washing prior to the co-culture with isolated islet cells. After various periods of time, islet cell lysis was monitored by trypan blue exclusion. At a target : effector cell ratio of 1 : 3, cytokine pre-activated IEC were found to lyse islet cells within 24 h (67.5 ± 4.2 %, $n = 4$; Fig. 1). Islet cells were protected from endothelium-mediated lysis when cultured in the presence of 0.5 mmol/l NMA. Protection was complete, i. e. observed islet death was identical to co-cultures with resident IEC (19.3 ± 11.9 vs 19.6 ± 11.1 %). Lysis correlated with the concentrations of the stable NO oxidation product nitrite found in the co-culture supernatants: after co-culture with pre-activated IEC high concentrations of nitrite (12.4 ± 2.4 nmol/l) could be measured, whereas in co-cultures with resident IEC or with activated IEC in the presence of NMA, respectively, only low nitrite concentrations were found (1.0 ± 0.6 vs 2.0 ± 1.2 nmol/l; Fig. 1). Culture of 3×10^4 purified islet cells in the absence or presence of the cytokine-mixture for 24 h resulted in the production of 2.0 ± 0.1 or 1.1 ± 0.5 ($n = 6$) nmol/l of nitrite. This is comparable to the value of 65 pmol nitrite per 2×10^3 fluorescence activated cell sorter (FACS) purified beta cells measured after incubation with IL-1β for 18 h as found by others [17]. Culture of islet cells for 24 h in the presence of the cytokine-mixture did not lead to increased lysis (10.6 ± 4.7 vs 8.9 ± 5.3 % lysis in the absence of cytokines, $n = 4$). Culture of cytokine-activated IEC in the presence or absence of islet cells did not significantly change the nitrite values found in the respective supernatants (data not shown).

To determine whether IEC-mediated islet cell lysis is beta cell-specific, we analysed co-cultures by electron microscopy. For the sake of quantification, these co-culture experiments were performed at a target : effector cell ratio of 1 : 1 under otherwise identical conditions. Culturing isolated islet cells with resident IEC or with pre-activated IEC in the presence of 0.5 mmol/l NMA resulted in the formation of small aggregates of intact islet cells (pseudo islets) strongly adherent to the IEC containing both beta and non-beta cells. In contrast, co-culture with pre-activated IEC led to complete lysis of both islet beta and islet non-beta cells after 24 h (Fig. 2) yielding single islet cells with all morphological aspects of lysis and no evidence for an apoptotic cell death (Fig. 3 A). Both beta and non-beta cells were protected from lysis by addition of 0.5 mmol/l NMA to values similar to control co-cultures with resident IEC.

To determine whether islet cell death induced by cytokine-activated IEC is preceded by DNA strand breaks as previously described for islet cell killing by activated macrophages [18], we also performed in situ nick-translation assays in these co-cultures. Indeed, DNA strand breaks were detected in 47.6 ± 7.3 % ($n = 3$) of islet cell nuclei 8 h after co-culture with activated IEC (Fig. 4 B). In contrast, when islet cells were co-cultured with resident IEC (Fig. 4 A), DNA strand breaks were a rare event (7.0 ± 2.4 %; $n = 3$). IEC never showed any DNA strand breaks.

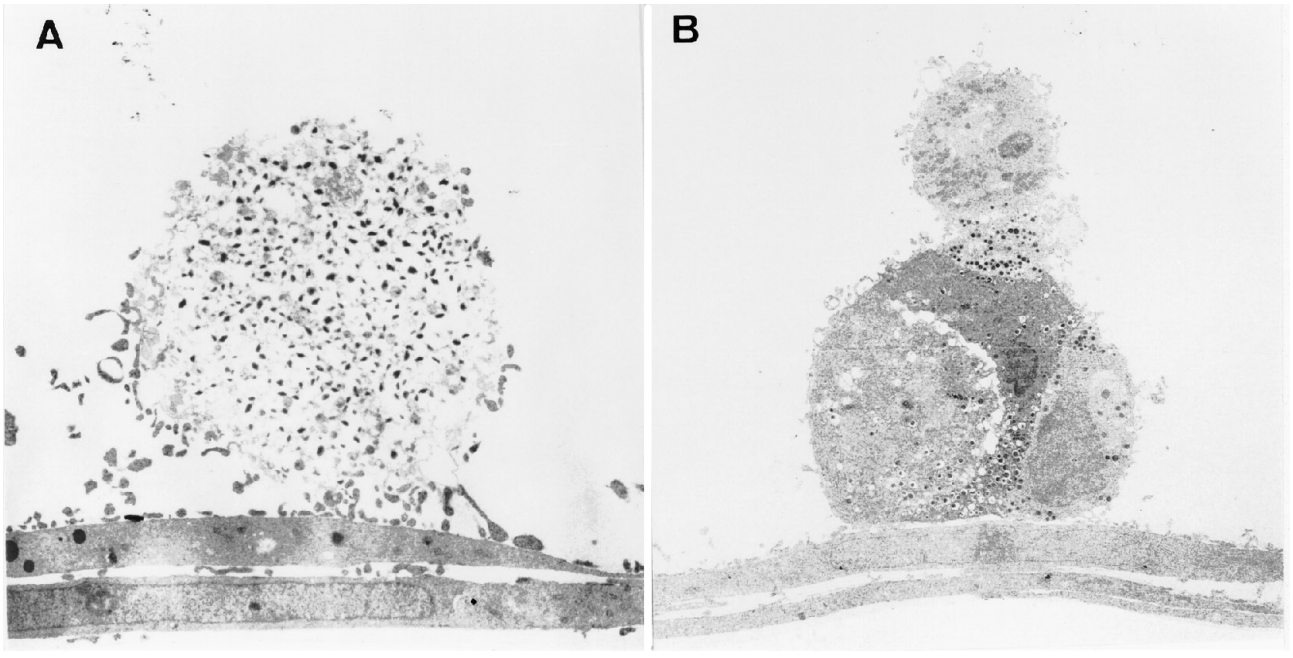

Fig. 3. (**A, B**) Electron micrographs of islet cells co-cultured with IEC. Isolated islet cells were cultured with pre-activated IEC at a target : effector cell ratio of 1 : 1 in the absence (**A**) or presence of 0.5 mmol/l NMA (**B**) for 24 h and were then processed for electron microscopy. Islet cell lysis is evident in cultures with pre-activated IEC as recognized by plasma membrane disruption and loss of cytoplasmic content among other features (**A**). In contrast, co-cultures with pre-activated EC in the presence of NMA resulted in normal islet cell morphology and the formation of small reassociated cell aggregates (**B**) with contact zones to the IEC exactly as found in co-cultures with resident IEC. Magnification A, ×5600; B, ×4000

Discussion

In animal models IDDM develops in the absence of MHC class I-restricted cytotoxicity, suggesting a contribution to tissue destruction by unspecific effector cells, e.g. macrophages and others [19]. There is increasing evidence from studies in animal models that iNOS expression in inflamed islets contributes to the destruction of islet cells via generation of high local concentrations of NO in addition to local production of oxygen radicals and pro-inflammatory cytokines.

Rat islets are highly capillarized [20, 21] with 8 to 10 beta cells forming a tube-like structure around a central capillary so that each beta cell faces two capillaries [22]. Thus, each beta cell within an islet is in proximity to two IEC. EC from larger vessels have been shown to be activated by cytokines to express iNOS and to produce large amounts of NO [4, 5]. Recently we demonstrated that this holds true for pure cultures of islet capillary EC as well [6]. Thus, activated endothelia in islets could theoretically become effector cells present in the inflammatory surrounding of infiltrated islets.

To test this hypothesis, we performed co-culture experiments. We found, that upon cytokine challenge IEC at the low target : effector cell ratios of 1 : 3 to 1 : 1 mediated strong to complete lysis of syngeneic islet cells via NO yielding single lysed islet cells still mostly in contact with the endothelial layer. With resident or NMA-treated activated IEC, islet cells formed small pseudo-islets and showed strong adherence of both beta and non-beta cells to IEC with broad contact zones suggesting the involvement of a specific adhesion mechanism. Pre-activated IEC were found to lyse both beta and non-beta cells, a result identical to previous data obtained for islet cell killing by activated macrophages [23]. Likewise, lysis of islet cells was preceded by DNA strand breaks with no morphological evidence of apoptosis identical to previous findings with activated macrophages [18] and NO donors [18, 24].

Activated EC are known to secrete cytokines such as IL-1β, IL-6 and IL-8 [1]. However, a previous study had shown that lysis of islet cells by activated macrophages was not inhibited or influenced by neutralizing antibodies specific for IL-1β or TNF-α [14]. Furthermore, the cytokine mixture used to pre-activate the IEC was not cytotoxic for the islet cells. Although IL-1β may severely impair cellular functions of islet cells [25], lysis of isolated islet cells due to incubation with IL-1β has not been demonstrated [26, 27] in contrast to lysis in whole islets [28, 29], suggesting the need of non-endocrine islet cells for IL-1β-mediated cytotoxicity. The combination of IL-1, TNF-α and gamma-IFN has been found to mediate partial cytotoxicity towards isolated islet cells but only after many days of culture [30, 31].

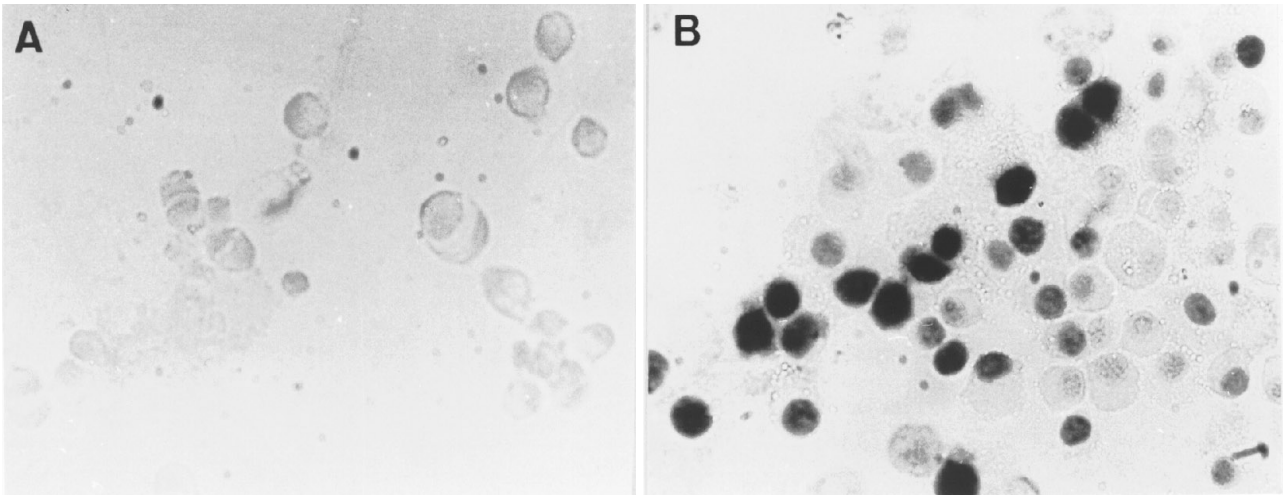

Fig. 4. (**A, B**) Detection of DNA strand breaks after co-culture with IEC. Isolated islet cells were co-cultured with resident (**A**) or pre-activated IEC (**B**) at a target : effector cell ratio of 1 : 3. After 8 h, cells were fixed and DNA strand breaks were detected by in situ nick translation. Micrographs were focussed to the plane of adhering islet cells with the endothelial layer below the foucs and only seen as a vague shadow, as EC nuclei did not stain. In contrast, islet cell nuclei show intense dark staining after co-culture with pre-activated IEC (**B**). Magnification × 560

In conclusion, we describe here for the first time that activated tissue-specific EC are able to lyse syngeneic mammalian target cells and to mediate DNA damage. IEC thus can actively participate in islet destruction and may thus be relevant effector cells in early stages of IDDM development as well as other inflammatory diseases.

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