Macrophage cytotoxicity towards isolated rat islet cells: neither lysis nor its protection by nicotinamide are Beta-cell specific

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Summary. In animal models of Type 1 (insulin-dependent) diabetes mellitus macrophages were shown to be the first immunocytes that infiltrate the pancreatic Langerhans islets in the autoimmune process. We now show direct macrophage cytotoxicity against isolated rat islet cells in an electron microscopical study, which permits investigation of the specificity of this process. Freshly isolated islet cells were co-incubated with syngeneic peritoneal macrophages at a target:effector-cell ratio of 1:2. After various time periods, the cells were directly fixed and embedded; the ratio of live and dead cells was evaluated by electron microscopy. Our results demonstrate that activated but not resident macrophages lyse islet cells in a time-dependent manner. After 15 h of co-incu-

bation lysis of islet cells is complete. No islet cell-macrophage contacts and no differences between the lysis of Beta cells or non-Beta cells were observed during the observation period. Islet cells encapsulated in alginate were also lysed by macrophages as a direct proof for soluble mediator(s) of cytotoxicity. Nicotinamide protected islet cells from lysis in a dose-dependent manner. As a result of this electron microscopical study we conclude that even at very low target:effector ratios, activated macrophages lyse syngeneic islet cells regardless of islet cell type via secretion of humoral mediator(s).

Key words: Islet cell lysis, macrophage cytotoxicity, nicotinamide.

It has been shown that macrophages are the major infiltrating immunocytes in the early stages of insulinitis in diabetes-prone BB rats [1, 2]. Furthermore, administration of silica particles, which are selectively toxic to macrophages, results in the prevention of insulinitis and diabetes in young BB rats [3]. A morphological study demonstrating macrophage cytotoxicity against islets and islet cell layer suggests a mechanism via direct cell-to-cell contacts between macrophages and islet cells [4]. Recently we showed that activated peritoneal macrophages lyse isolated ³H-leucine labelled syngeneic islet cells but not thyrocytes or hepatocytes [5].

Nicotinamide at high doses inhibits poly(ADP-ribose) synthetase activity [6, 7], but may also scavenge hydroxyl free radicals [8, 9]. Nicotinamide has been shown to prevent induction of diabetes by alloxan or streptozotocin [10, 11] in the rat and to improve the condition of partly depancreatized rats [12]. Furthermore, nicotinamide prevents or slows the manifestation of diabetes in non-obese diabetic (NOD) mice [13, 14].

This study describes the morphological results of in vitro experiments designed to distinguish between phagocytosis, lysis by direct cell-to-cell contact or without cell contact at a low target:effector-cell ratio (1:2). In addition, the Beta-cell specificity and the protective action of nicotinamide is analysed.

Materials and methods

Animals

Male and female Wistar and Lewis rats (200 g) from a local breeding source received a standard diet ("ssniff-R", Ssniff, Soest, FRG) and tap water ad libitum.

Target and effector cells

Islet cells and peritoneal macrophages were isolated as described previously [5]. Briefly, pancreatic islets were isolated by ductal injection of collagenase (Serva, Heidelberg, FRG, 0.32 U/mg, 3 mg/ml in Hank's buffered saline, HBSS) and subsequent centrifugation of the dispersed tissue on a Ficoll density gradient (Ficoll 400, Pharmacia, Freiburg, FRG). Whole islets were hand-picked and incubated for 10 min (37°C; 5% CO₂) in Ca/Mg-free HBSS with 3 mmol/l EGTA (Merck, Darmstadt, FRG), 20 mg/ml bovine serum albumin, 11 mmol/l glucose and 2.5 mg/ml trypsin solution (Boehringer, Mannheim, FRG), final dilution 50 µl/ml, and subsequently dissociated into single cells. The islet cell preparations contained less than 5% exocrine pancreatic cells or endothelial cells or fibroblasts.

Macrophages were activated in vivo by injection of 500 µl heat inactivated *Corynebacterium parvum* suspension (Wellcome, Burgwedel, FRG) i.p. 5 days prior to isolation. Macrophages from peritoneal exudate were isolated by adherence to petri dishes pre-coated with fetal calf serum (FCS). Adherent cells were rigorously washed three times with 1 h culture period between washings. Adherent

Fig. 1a, b. Low power electron micrographs of islet cells after 8 h of recovery culture and 15 h of culture in the absence (a) or presence (b) of activated macrophages. **a** Islet cells of control culture show rearrangement to pseudoislets with normal, well granulated cells (B Beta islet cells, A Alpha islet cells). **b** Islet cells cultured as in a but in the presence of activated macrophages at a target:effector-cell ratio of 1:2. All islet cells are lysed. The arrow points to a macrophage engaged in Beta-islet cell debris uptake. Magnification: 3250 ×

cells were harvested by incubation in Ca/Mg-free HBSS at 4°C for 5 min. As judged by morphology, the resulting cell preparations consisted of 92% macrophages, 6% granulocytes, 1.5% mast cells and less than 0.5% lymphocytes, which is in accordance with published cytochemistry results [5].

Co-culture experiment with isolated islet cells

About 5×10^4 target cells in 200 µl were placed in sterile 1.5 ml polypropylene micro sample tubes with V-shaped bottom that had been pre-coated with culture medium for 48 h at 37°C. After 3–8 h of recovery culture, peritoneal macrophages were carefully added into the sample tubes with the pre-cultured islet cells to achieve a target:effector-cell ratio of 1:2 and a total volume of 600 µl. The final co-culture was neither stirred nor centrifuged, but cells were allowed to settle. The cells were cultured in RPMI 1640 (Gibco, Heidelberg, FRG)

supplemented with 25 mg/l ampicillin, 120 mg/l penicillin, 270 mg/l streptomycin (Serva), 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 10 ml/l non-essential amino acids × 100 (Gibco), 23.8 mmol/l NaHCO₃, 10 mmol/l HEPES (Serva) and 10% heat inactivated FCS (Boehringer) for various intervals at 37°C in a humid atmosphere of 5% CO₂/95% air. Each experiment was performed between two and six times.

Co-culture experiments with alginate-encapsulated islet cells

Alginate, a linear polysaccharide composed of β-D-mannuronic and α-L-guluronic acid, can be gelled by divalent cations and has been used to immobilize living cells [15].

About 5×10^4 islet cells were cultured for 5 h in microsample tubes as described above. The culture medium was then reduced to 2–

Fig. 2a, b. Electron micrographs of phagocytosing macrophages in co-culture with islet cells after 3 h (a) or 8 h (b) recovery culture. **a** Phagocytosis of whole islet cells (★) by macrophages was a frequent event, if recovery culture lasted 3 h only and is indicative for recognition of trypsin-altered islet cell surface. **b** Extending islet cell recovery culture to 8 h prior to macrophage co-culture, no phagocytosis of whole intact cells can be observed, but macrophages (*mØ*) are engaged in the uptake of debris from previously lysed islet cells. Magnification: 8000 ×

Fig. 3. Electron micrograph of co-cultured cells in the presence of nicotinamide. Macrophage-mediated lysis of islet cells is completely prevented by the addition of 50 mmol/l nicotinamide. Morphology of islet cells is well preserved with normal stacks of rough endoplasmic reticulum (arrow) even in close contact with macrophages (*mØ*) (B Beta islet cell, A Alpha islet cell). Magnification: 8400 ×

3 µl · 5 µl of a degassed alginate stock solution (4% sodium alginate of low viscosity from *Macrocystis pyrifera* (Sigma, Deisenhofen, FRG) dissolved (weight/volume) in Ca/Mg-free HBSS containing 10 mmol/l HEPES) was added and the cells were carefully suspended. Then 2 µl droplets of the cell suspension were added to 1 ml HBSS containing 10 mmol/l HEPES and 50 mmol/l CaCl₂, pH 7.3, allowed to gelate for 10 min and then washed with 1 ml RPMI/10% FCS. Single alginate beads containing 1–2 × 10⁴ islet cells were incubated in 200 µl RPMI/10% FCS for 2 h at 37°C for recovery from the entrapment procedure. Medium or activated macrophages were added to give a final volume of 400 µl. The cells were cultured for 15 h as described above.

Electron microscopy

Fixation, dehydration and embedding of the cells were all carried out in the micro sample tubes in which the cells had been cultured avoiding any centrifugation steps. The cells were fixed with ice-cold fixa-

tion buffer (1% glutaraldehyde in 0.1 mol/l cacodylate-HCl buffer, 0.1 mol/l sucrose, and 2 mmol/l CaCl₂, pH 7.2) for 10 min, post-fixed with ice-cold 1.5% OsO₄ in the same buffer for 40 min, dehydrated in a graded series of ethanol and embedded in epoxy resin [16]. The embedded specimen were processed for transmission electron microscopy as described earlier [17].

Results

Morphology of the islet cells

Islet cells were embedded either directly after isolation, following 3 or 8 h of recovery culture and an additional 15 h of culture in micro sample tubes. We found that under these culture conditions the islet cells re-associated in a time-dependent fashion and formed small cell aggregates

Fig. 4. Quantitative determination of lysed vs intact Beta islet cells (left panel, $n = 2575$) and non-Beta islet cells (right panel, $n = 628$) after 8 h recovery culture and various times of co-incubation with activated macrophages (1:2) shows a time dependent lysis of both, Beta as well as non-Beta islet cells. Dotted bars: intact islet cells, hatched bars: lysed islet cells. (A: control incubation, 15 h, no macrophages added B: 4 h co-incubation C: 8 h co-incubation D: 12 h co-incubation E: 15 h co-incubation)

Fig. 5. Quantitative determination of lysed vs intact Beta islet cells (left panel, $n = 1814$) and non-Beta islet cells (right panel, $n = 410$) after 8 h recovery culture and co-incubation with resident or activated macrophages (1:2) for 15 h in the presence of different glucose concentrations. (A: co-incubation with resident macrophages, 4 mmol/l glucose B: co-incubation with resident macrophages, 11 mmol/l glucose C: co-incubation with activated macrophages, 4 mmol/l glucose D: co-incubation with activated macrophages, 11 mmol/l glucose)

with well preserved and well granulated endocrine cells. After about 24 h of culture, pseudoislets were formed with Alpha and Delta cells mostly located at the periphery (Fig. 1 a). The cellular composition and the non-random distribution of Beta cells and non-Beta cells were similar to that found in pseudoislets received under different culture conditions [18, 19] and in intact islets [20].

At the light microscopical level cell viability as determined by Trypan blue exclusion demonstrated that after 24 h of culture under the conditions used for the electron microscopical studies more than 90% of the islet cells were intact, most of them re-arranged to cell-clusters.

On the electron microscopical level it was found that after 24 h of culture more than 95% of the Beta cells and about 70–80% of the non-Beta cells were intact.

Co-culture of islet cells with macrophages

After co-culturing islet cells and activated macrophages in close proximity at the bottom of micro sample tubes, three different effects could be defined: a) lysis of islet cells, b) phagocytosis of previously lysed islet cells and c) phagocytosis of intact islet cells. Lysis of islet cells is characterized by disrupted plasma membranes concomitant with low electron density of the cytoplasm, swollen mitochondria, vesiculated rough endoplasmatic reticulum and pyknotic

nuclei (Fig. 1 b). Phagocytosis of previously lysed or intact cells could clearly be distinguished: after phagocytosis of previously lysed cells debris was found in many separate macrophage vacuoles, while after phagocytosis of whole islet cells these were found within one macrophage vacuole (Fig. 2 a, b).

When islet cells were co-incubated with activated macrophages after 3 h of recovery culture following isolation, many intact islet cells were phagocytosed by the activated macrophages. Prolonging the recovery culture to 8 h (when islet cell aggregates could already be detected), less than 0.5% of the islet cells were phagocytosed as intact cells. This was taken as an indication that a 3 h recovery period was not sufficient for islet cells to repair trypsin-induced alterations. Macrophages apparently recognize the altered-self and phagocytose these cells. After a prolonged recovery period minimal phagocytosis was interpreted as an indicator of complete islet cell surface repair. Therefore, a recovery culture of 8 h was taken as standard condition.

Time-dependent lysis of islet cells by activated macrophages

Islet cells and peritoneal macrophages which had been activated *in vivo* by application of heat-inactivated *Corynebacterium parvum* were co-cultured for 4, 8, 12 and

Fig. 6. Quantitative determination of lysed vs intact islet cells ($n = 2633$) entrapped within alginate beads after 5 h recovery culture. Encapsulated islet cells were again cultured for 3 h, and then co-incubated for 15 h with activated macrophages at different target:effector-cell ratios for 15 h. (A: control incubation without macrophages B: co-incubation with activated macrophages (1:2) C: co-incubation with activated macrophages (1:10))

Fig. 7a, b. Low power electron micrographs of alginate-encapsulated islet cells after 8 h recovery culture and 15 h of culture in the absence (a) or presence (b) of activated macrophages. **a** Alginate-encapsulated islet cells in control cultures show normal, well granulated morphology. **b** Culture as in **a** but in the presence of activated macrophages at a target:effector-cell ratio of 1:10 results in almost complete lysis of the encapsulated islet cells. The grayish material surrounding pseudoislets represents the alginate meshwork (B: Beta islet cells, A: Alpha islet cells). Magnification: 3250 $\times$

Fig. 8. Quantitative determination of lysed vs intact Beta islet cells (left panel, $n = 3915$) and non-Beta islet cells (right panel, $n = 1169$) after 8 h recovery culture and co-incubation with activated macrophages (1:2) for 15 h in the presence of various concentrations of nicotinamide. (A: control incubation, no macrophages and no nicotinamide added B: control incubation, no macrophages, 50 mmol/l nicotinamide C: co-incubation with macrophages, no nicotinamide D: co-incubation, 5 mmol/l nicotinamide E: co-incubation, 25 mmol/l nicotinamide F: co-incubation, 50 mmol/l nicotinamide)

15 h, respectively, in the presence of 11 mmol/l glucose. Parallel islet cells were cultured under the same conditions without macrophages as control. Figure 4 shows that co-culturing islet cells and activated macrophages resulted in a time-dependent lysis of both Beta and non-Beta cells. While almost all islet cells are intact after 4 h of co-incubation, only 20–30% of the islet cells were morphologically intact after 8 h of co-culture. After 15 h target cell lysis was complete (Fig. 1b). No predominance of Beta or non-Beta cell lysis, no cell-to-cell contacts between macrophages and intact islet cells and no significant phagocytosis of intact target cells could be detected in any of the co-culture periods investigated.

Co-culture of islet cells with resident or activated macrophages

To investigate the influence of islet cell stimulation by glucose and the activation status of the macrophages on target-cell lysis, islet cells were co-incubated with resident and activated macrophages, respectively, in the presence of 4 and 11 mmol/l glucose. Figure 5 shows that after 15 h of co-incubation with resident macrophages, more than

80% of the Beta cells and more than 70% of the non-Beta cells remained intact while in the same time more than 90% of the islet cells were lysed by activated macrophages. No difference between the experiments with 4 and 11 mmol/l glucose could be found. Similar results were observed with both Wistar and Lewis rats.

Co-culture of alginate-encapsulated islet cells with activated macrophages

To exclude direct cell-to-cell contacts between macrophages and islet cells and to investigate whether microencapsulation of islet cells protects from macrophage-mediated killing, the targets were encapsulated in small alginate beads. The entrapped islet cells cultured in the absence of macrophages were well preserved and well granulated. The cellular composition and the non-random distribution of Beta cells and non-Beta cells were the same as found in the experiments described above. About 95% of the islet cells were intact after 15 h of culture (Figs. 6 and 7a). No islet cells outside the alginate beads could be detected, indicating, that the beads were stable during the incubation and embedding procedure. Co-incubation of the encapsulated islet cells with activated macrophages for 15 h at a target:effector-cell ratio of 1:2 resulted in lysis of about 40% of the islet cells (Fig. 6). When the target:effector-cell ratio was raised to 1:10, almost complete lysis was evident after 15 h of co-culture (Fig. 6). Cell lysis of alginate encapsulated and non-encapsulated islet cells was indistinguishable by morphology (Fig. 7b). No macrophages could be detected inside the alginate beads nor were they found adhering to the bead surface. Encapsulation of activated macrophages and subsequent co-incubation with isolated islet cells also resulted in lysis of target cells (data not shown).

Protective effect of nicotinamide

To investigate if nicotinamide protects islet cells from lysis by activated macrophages, the co-culture experiments were done in the presence of various concentrations of nicotinamide. Figure 8 shows that 5 mmol/l nicotinamide did not protect islet cells from lysis by activated macrophages, while in the presence of 25 mmol/l nicotinamide, most Beta cells (79.0%) and many non-Beta cells (35.7%) remained intact. 50 mmol/l nicotinamide results in almost complete protection of the target. In the presence of 50 mmol/l nicotinamide both activated macrophages and islet cells were morphologically indistinguishable from controls (Fig. 3). No islet cell-macrophage contacts were present in these experiments. Pre-incubation of activated macrophages with 50 mmol/l nicotinamide for 1 h and subsequent co-culture in the absence of nicotinamide did not protect islet cells (data not shown). Similar results were observed with both Wistar and Lewis rats.

Discussion

We recently reported that activated macrophages spontaneously lyse pancreatic islet cells [5]. In these experiments the islet cells were incubated with radioactive ^3H -leucine

prior to co-culture with macrophages. This experimental setup does not permit distinction between Beta and non-Beta islet cells and uses a high target:effector-cell ratio of 1:20–40 which is necessary to detect significant radioactive label release.

We now have developed an experimental setup with a low target:effector-cell ratio (1:2) examining the cells after co-culture experiments by electron microscopy. The main difference to the procedure described before [5] is, that islet cells and macrophages are now forced into comparatively close, “tissue-like”, proximity by the V-shaped bottom of the sample tubes. This appears to allow for efficient diffusion of mediator(s) to target cells compared to flat-bottom well incubation with comparatively long diffusion distances.

The results obtained clearly show that co-incubation of activated macrophages with isolated islet cells results in a time-dependent lysis of the target cells. No differences in the sensitivity of Beta or non-Beta cells against macrophage-mediated lysis could be detected. However, it should be noted that non-Beta cells, which are mostly located at the periphery of the pseudoislets, are more sensitive to the isolation procedure and the culture conditions than the Beta cells. This in vitro situation is the reverse of the in vivo situation, where soluble mediators transported via blood would reach Beta cells first, due to the direction of blood supply [21].

In all experiments a basic finding was that no cell-to-cell contacts between activated macrophages and intact islet cells were formed. This indicates that macrophage cytotoxicity does not require cell-to-cell contacts with islet cells but is mediated by one or several secreted macrophage products. This finding is reminiscent of macrophage cytotoxicity towards murine P815 mastocytoma cells [22, 23].

Direct proof for the cytolytic action of (a) humoral macrophage product(s) was provided by the encapsulation experiments, where both encapsulation of either targets or effectors invariably resulted in islet cell lysis. These experiments show that the toxic agent(s) can diffuse over a comparatively large distance (up to 700 μm to the interior of the alginate bead) but that under these conditions higher concentrations of the cytolytic product(s) are required for complete lysis as reflected by the need for a higher target:effector ratio.

The macrophage product interleukin-1 has been shown to exert a cytolytic effect on islets when administered for up to 6 days [24]. The combination of interleukin-1 and tumour necrosis factor was found to synergistically affect isolated islet cells after co-incubation for 4 days [25]. However, islets treated with interleukin-1 showed reduced glucose-induced insulin secretion, but were morphologically intact and regained their ability to secrete insulin after the removal of interleukin-1 [26]. We show here that diffusible macrophage product(s) mediate rapid islet cell killing within 15 h. Due to the fast action found here other cytolytic secretory products of macrophages such as reactive nitrogen or oxygen intermediates and neutral proteases [27] may be responsible for islet cell lysis.

This was further supported by the finding that high concentrations of nicotinamide, which are not toxic to

islet cells or macrophages, completely prevented both Beta and non-Beta cells from lysis by activated macrophages. Earlier experiments with nicotinamide in co-cultures of macrophages and radiolabelled islet cells have also shown protective effects [28]. It has been shown that high doses of nicotinamide scavenge hydroxyl free radicals [8]. It was also found that nicotinamide prevents DNA single-strand breaks in rat insulinoma cells when cultured in the presence of alloxan [9], but when cultured in the presence of streptozotocin, nicotinamide increased DNA single-strand breaks in the same cells indicative of inhibition of the poly(ADP-ribose) synthetase [9]. With the data obtained in our experiments we cannot distinguish which of these nicotinamide effects exerts the protection from lysis. However, the long distance of action found in the encapsulation experiments are not in favour of short-lived radicals as the main active product. We conclude that macrophage cytotoxicity towards islet cells *in vitro* is not Beta-cell specific, is mediated by soluble factor(s) and is counteracted by nicotinamide.

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