*Rapid communication***CD95 and CD95L expression in endocrine pancreas of NOD, NOR and BALB/c mice****A. Signore¹, A. Annovazzi¹, E. Procaccini¹, P. E. Beales², J. Spencer³, R. Testi⁴, G. Ruberti⁵**¹ Nu.M.E.D. Group, Clinica Medica II, University “La Sapienza”, Rome, Italy² Department of Diabetes and Metabolism, St. Bartholomew's Hospital, London, UK³ UMDS, St. Thomas' Hospital, London, UK⁴ Dipartimento di Medicina Sperimentale, University “Tor Vergata”, Rome, Italy⁵ Department of Immunobiology, Institute of Cell Biology, National Research Council, Rome, Italy

Summary The non-obese diabetic (NOD) mouse is widely used to study the pathogenesis of insulin-dependent diabetes mellitus. However, the mechanisms responsible for beta-cell destruction, in this model, are still poorly defined. The CD95/CD95L system among other effector systems has been implicated in beta-cell death. In this study we investigated in NOD, non-obese resistant (NOR) and Balb/c mice

the expression of CD95 and CD95L in alpha and beta pancreatic cells by immunohistochemistry and immunofluorescence. We demonstrate that alpha cells in the islets of Langerhans constitutively express CD95L forming a natural shield around beta cells. [Diabetologia (1997) 40: 1476–1479]

Keywords Apoptosis, CD95, CD95L, NOD mouse.

The non-obese diabetic (NOD) mouse spontaneously develops a disease with many of the features of human insulin-dependent diabetes mellitus (IDDM) and is widely used to investigate the pathogenesis of this autoimmune disease. In NOD mice a progressive infiltration of the islets of Langerhans by lymphoid and myeloid cells called insulinitis is followed by massive destruction of insulin producing beta cells, eventually leading to diabetes [1]. The mechanism by which beta cells die has yet to be elucidated. Data from several experimental models implicate different death effector systems, such as CD95/CD95L interaction [2, 3], perforin and tumour necrosis factor (TNF)/TNFR1 interaction [4], in beta-cell destruction

Binding of CD95 (also known as Fas or APO-1) by its ligand (CD95L) commonly induces apoptosis of CD95 positive cells. CD95 is constitutively expressed in a wide range of non-immune cells and is also inducible in some lymphoid and non-lymphoid cell types upon appropriate stimulation [5]. CD95L expression

in some tissues may induce cell death of potential dangerous infiltrating lymphoid cells [6, 7]. It can therefore be postulated that lack of CD95L expression may facilitate tissue destruction by autoreactive T cells and that, conversely, its expression might confer immune privilege to particular cell types. Thus, CD95-CD95L interaction is part of a complex interplay of different cell types resulting either in killing or protection of target cells.

We investigated the expression of CD95 and CD95L in the pancreatic islets of NOD mice and also of Balb/c and non-obese resistant (NOR) mice of different ages and sex, in view of a possible role for the CD95/CD95L system in the beta-cell damage resulting in diabetes.

Materials and methods

Animals. The St. Bartholomew's Hospital NOD colony (NOD/Ba) was established in 1987. Mice were originally obtained from Dr. Leiter's laboratory (Bar Harbor, Maine, USA). There is a stable cumulative diabetes incidence of 55 % in females and 15 % in males [1].

We killed NOD/Ba mice, 15 males and 15 females, by neck dislocation at the following ages: 4–8 weeks (3 males and 3 females), 12 weeks (3 males and 3 females), at 20 weeks (3 males and 3 females), 39–42 weeks (3 males and 3 females) and 6 re-

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Abbreviations: NOD, Non-obese diabetic; IDDM, insulin-dependent diabetes mellitus; NOR, non-obese resistant.

Fig. 1 A–E. Pancreatic cryostat sections of NOD (**A, B, D, E**) and Balb/c (**C**) mice showing the expression of CD95L on alpha cells. In **A** and **B**, two consecutive sections were stained to reveal the presence of CD95 and CD95L respectively, in an islet infiltrated by mononuclear cells (“destructive” pattern). In **C**, the section is stained to reveal the presence of CD95L and shows a typical “complete” ring of CD95L positive cells in contrast with the “non-complete” ring shown in **B**. In **D** and **E** the same section shows the presence of CD95L (**D**) expressed only on glucagon containing cells (**E**). Only one glucagon containing cell can be detected as CD95L negative (arrow)

cently diagnosed diabetic mice (3 males and 3 females). These were killed within a week from the onset of diabetes as defined by the presence of glycosuria greater than 55 mmol/l for more than 1 week (measured by Diaburstix; Boehringer Mannheim, Mannheim, Germany). Furthermore, we studied 12 Balb/c mice, 6 males and 6 females at different ages (6, 20 and 40 weeks) and 12 NOR (non-obese resistant) mice (6 males and 6 females) at different ages: 8 weeks (1 male and 1 female), at 16 weeks (2 males and 2 females), at 24 weeks (2 males and 2 females).

The pancreas was removed, frozen in liquid nitrogen and stored at -80°C until histological study was performed.

Histological analysis. Cryostat sections were prepared as follows: 4 consecutive 6 μm sections were cut and put on 4 different slides; approximately every 250 μm another 4 consecutive sec-

tions were cut and put on the slides, until each slide had 3 different sections thus allowing 10 to 30 islets of Langerhans for each pancreas to be evaluated. One slide was used for CD95 immunostaining, the second one for CD95L, the third as negative control and the fourth was stained with haematoxylin-eosin. Sections were examined blindly by two different readers.

Immunoperoxidase staining. Sections for immunoperoxidase staining were fixed in fresh acetone for 30 min at room temperature. Endogenous biotin was blocked using a Streptavidin-Biotin blocking Kit (VECTOR sp-2001, Burlingame, Ca, USA). Then sections were stained with the following antibodies: biotinylated (Rat IgM) anti-mouse CD95L MoAb (Alexis 804-009B-T100, San Diego, Ca., USA) 1:50 in Tris Buffered Saline (TBS) with 10% normal mouse serum and (rabbit) anti-mouse CD95 polyclonal antibody (Santa Cruz Biotechnology sc-716;

Fig. 2. Correlation, in NOD mice, between the “severity of insulinitis”, as percentage of islets with a “destructive” insulinitis pattern (histologically evaluated) and CD95L expression, as percentage of islets with a “complete” ring of CD95L positive cells (immunohistochemically evaluated)

Santa Cruz, Ca, USA) 1:500 in 10 % normal mouse serum. After 1 h incubation 30 ml of biotinylated rabbit anti-rat immunoglobulins (Dako E0468; Glostrup, Denmark, 1:300 TBS) or biotinylated goat anti-rabbit immunoglobulins (Dako E0432, Glostrup, Denmark 1:600 TBS) were added to the sections, respectively, and left for 60 min. The slides for negative control were stained only with the second antibodies. At the end of incubation all sections were treated with streptavidin ABC complex (Dako K0377) for 30 min and, after a 10 min incubation with diaminobenzidine substrate (1 mg/ml in Tris-HCl buffer), they were counterstained with Mayer’s Haematoxylin.

All antibodies were previously tested and titrated on Balb/c spleen (positive control for CD95) and testis sections (positive control for CD95L).

Following examination of CD95L expression in the islets of Langerhans, we scored each islet as having a “complete” or a “non-complete” ring of CD95L positive cells. We then calculated for each NOD mouse the percentage of islets with a “complete” ring (Fig. 1).

Immunofluorescence staining. For double immunofluorescence studies, other cryostat sections were cut, fixed in acetone for 30 min and stained as follows: alpha and beta cells were stained using a glucagon or insulin guinea pig anti-mouse antiserum (kindly donated by Dr. G. Cavallo) and revealed by 1 h incubation with a fluorescein isothiocyanate (FITC) conjugated rabbit anti-guinea pig polyclonal antibody (Sigma F7762; St. Louis, Mo, USA). The second staining was performed using a biotinylated CD95L anti-mouse MoAb (Rat IgM) revealed by 30 min incubation with Cy3-labelled-extravidin (Sigma, E4142). Before adding the CD95L MoAb, sections were incubated for 10 min with 10 % normal rat serum to avoid non-specific binding of the CD95L MoAb.

For double CD95 and glucagon or insulin staining we performed the following incubations: CD95 polyclonal antibody (1 h), biotinylated goat anti-rabbit immunoglobulins (30 min), Cy3-labelled-extravidin (30 min), 10 % normal rabbit serum (10 min), glucagon or insulin guinea pig antiserum (1 h), FITC conjugated rabbit anti-guinea pig polyclonal antibody (1 h).

Sections were then examined under an ultraviolet microscope using two different filters to visualize alternatively fluorescein (525 nm) and Cy3 (570 nm).

Morphological analysis of insulinitis. The “extent of insulinitis” was evaluated and scored for each islet as previously described [1]. Briefly, islets were scored as 0 if no detectable lymphocytes were in the islet; 1) when few lymphocytes were around the islet (peri-insulinitis); 2) if less than 50 % of islet area was infiltrated by lymphocytes; 3) if more than 50 % of islet area was infiltrated by lymphocytes. The score of the extent of insulinitis was calculated for each pancreas.

Furthermore, the “severity of insulinitis” was scored as the percentage of islets with a “destructive” pattern. This was defined as the presence of mononuclear cells detectable inside the islet with loss of its architecture in contrast with the “non-destructive” pattern, defined as a mononuclear cell infiltrate surrounding the islet without loss of its architecture.

Only islets with insulinitis score of 2 or 3, where then scored for severity, since all peri-insulinitis (extent score = 1) is non-destructive by definition. Approximately 3 % of islets were not considered for the “severity” score as they appeared with a borderline pattern.

Results

CD95 was not detected in Balb/c pancreas or in non-infiltrated NOD or NOR mouse islet cells. However, strong expression of CD95 appeared on mononuclear cells infiltrating the islets of NOD and NOR mice (Fig. 1 A).

By contrast, CD95L was expressed on the endocrine pancreas of all mouse strains. Interestingly, positive cells were located in the periphery of islets forming a ring around them (Fig. 1 B and C). Only a few CD95L positive cells appeared in the middle of the islets. CD95L was undetectable on the mononuclear cells infiltrating the NOD and NOR islets.

Remarkably, double immunofluorescence studies revealed in all mouse strains that all CD95L positive cells were also positive for glucagon, indicating that they were alpha cells (Fig. 1 D and E).

Moreover, in NOD mice a “complete” or a “non-complete” ring of CD95L positive alpha cells around beta cells correlated with a “non-destructive” or “destructive” insulinitis pattern (Fig. 2). No correlation was observed between the “extent” of insulinitis and the pattern of CD95L expression in the islets.

By contrast, insulin positive beta cells did not show detectable levels of CD95 or CD95L expression in any mice tested (data not shown).

Discussion

Here we show that pancreatic alpha cells of NOD, NOR and Balb/c mice constitutively express CD95L and that, within the limits of discrimination of immunohistochemical analysis, no quantitative difference in the expression was seen in the different strains of mice. The expression of CD95L on alpha cells might play opposing roles in the evolution of diabetes in NOD mice at different stages or under different

microenvironmental conditions: a protective or an enhancing effect.

As far as a possible protective role is concerned, the islets of Langerhans can be considered an immunologically privileged tissue like the testis and the cornea [6, 7]. CD95L expression on alpha cells may act as a shield to protect beta cells from immunological attack. A similar protective effect on beta cells has been observed when CD95L expressing syngeneic myoblasts were co-transplanted with allogeneic islets in mice made diabetic with streptozotocin [8]. Nevertheless, the analysis of histological sections showed that in some islets mononuclear cells may have overcome this protection and penetrated the alpha-cell barrier (non-complete alpha-cell ring), resulting in a "destructive" insulitis with beta-cell destruction. In other islets, mononuclear cells would not penetrate into the CD95L positive alpha-cell barrier (complete alpha-cell ring) and this would be morphologically characterized by a typical "non-destructive" insulitis pattern, without destruction and loss of beta-cell mass.

On the other hand, CD95L expression on alpha cells might play a promoting role in the evolution of diabetes by contributing to beta-cell killing. It has been demonstrated that interleukin (IL)-1 beta induces CD95 expression in human thyrocytes and that co-expression of functional CD95 and CD95L in Hashimoto's thyroiditis may contribute to thyroid destruction [9]. CD95 expression has also been described on isolated human beta cells after IL-1 stimulation [10]. While we were unable to detect CD95 expression by immunostaining on murine beta cells it is possible that during IDDM progression in NOD mice CD95 expression is upregulated on beta cells to levels that make them sensitive to CD95L but which are too low to be detected by immunohistochemical staining, as also suggested by the results obtained in the CD95L transgenic mice [2]. If this is the case, CD95L positive alpha cells might be responsible for beta-cell destruction at some stage of the disease.

A better understanding of how mononuclear cells penetrate the alpha-cell barrier and how pancreatic

alpha and beta cells interact, may provide new insights in clarifying the pathogenesis of IDDM.

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