

Is pancreas development abnormal in the non-obese diabetic mouse, a spontaneous model of type I diabetes?

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Abstract

Despite extensive genetic and immunological research, the complex etiology and pathogenesis of type I diabetes remains unresolved. During the last few years, our attention has been focused on factors such as abnormalities of islet function and/or microenvironment, that could interact with immune partners in the spontaneous model of the disease, the non-obese diabetic (NOD) mouse. Intriguingly, the first anomalies that we noted in NOD mice, compared to control strains, are already present at birth and consist of 1) higher numbers of paradoxically hyperactive β cells, assessed by *in situ* preproinsulin II expression; 2) high percentages of immature islets, representing islet neogenesis related to neonatal β -cell hyperactivity and suggestive of *in utero* β -cell stimulation; 3) elevated levels of some types of antigen-presenting cells and FasL⁺ cells, and 4) abnormalities of extracellular matrix (ECM) protein expression. However, the colocalization in all control mouse strains studied of fibroblast-like cells (anti-TR-7 labeling), some ECM proteins (particularly, fibronectin and collagen I), antigen-presenting cells and a few FasL⁺ cells at the periphery of islets undergoing neogenesis suggests that remodeling phenomena that normally take place during postnatal pancreas development could be disturbed in NOD mice. These data show that from birth onwards there is an intricate relationship between endocrine and immune events in the NOD mouse. They also suggest that tissue-specific autoimmune reactions could arise from developmental phenomena taking place during fetal life in which ECM-immune cell interaction(s) may play a key role.

Key words

- NOD mice
- Postnatal pancreas development
- Antigen-presenting cell
- Extracellular matrix

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Introduction

Type I diabetes is one of the most frequent chronic diseases of children caused by the autoimmune destruction of pancreatic insulin-producing β cells (insulinitis) (1). Because human pancreatic tissue is unavailable, our present understanding of the pathogenesis of type I diabetes has been made possible over the past two decades by the existence of two spontaneous animal models

of the disease: the BioBreeding rat and the non-obese diabetic (NOD) mouse (1,2). The immune reaction against β cells occurs on a multigenic susceptibility background, while environmental and hormonal factors modulate disease development (1-3). However, despite major progress in the elucidation of genetic and immune mechanisms, several issues of theoretical significance remain unresolved, among which perhaps the most important one, i.e., the initial trigger for break-

ing self tolerance, is unknown.

NOD mice develop autoimmunity towards islets of Langerhans around weaning (3 weeks of age). The results of many studies have implicated defective regulation of immune function (2). This explanation, however, does not take into account that the autoimmune process is strongly organ specific. While abnormalities of immune regulation may explain the predisposition to autoimmunity in general, additional factors probably determine the specific target of the autoimmune attack and, particularly, the target organ itself. We hypothesized that defects in islet differentiation and/or function could drive the immune system towards islet autoimmunity (4) and therefore investigated various immune and endocrine parameters during the early prediabetic stage in NOD mice.

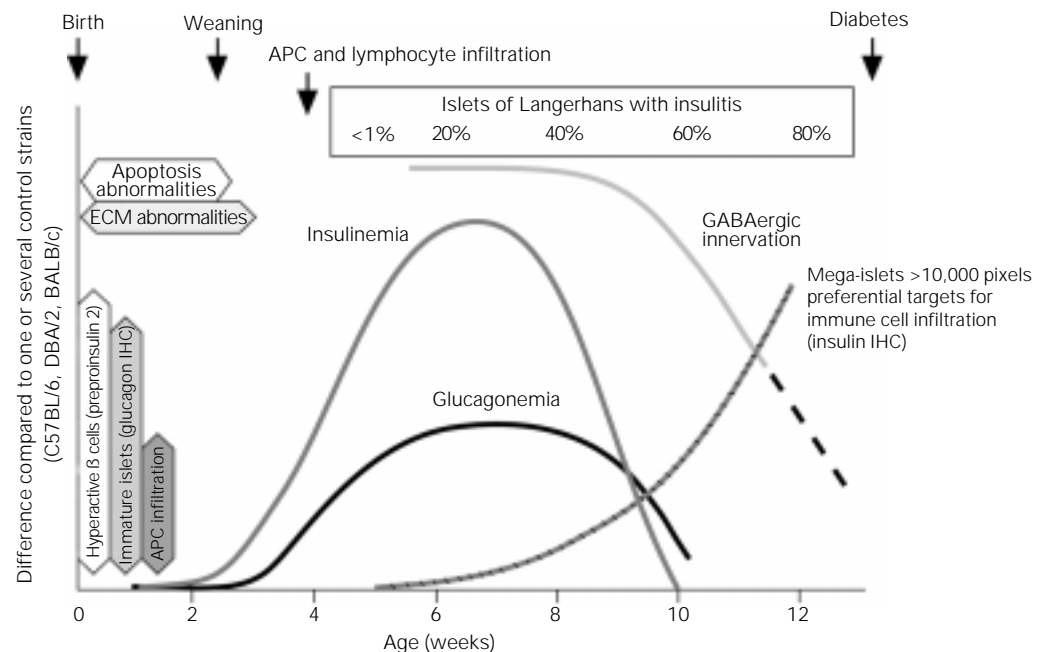
Antigen-presenting cell infiltration and islet abnormalities in prediabetic NOD mice

We showed that in wild-type NOD mice the first sign of infiltration is an accumula-

tion of CD11c⁺ dendritic cells (DC) around the islets from about 3-4 weeks onwards, i.e., notably around weaning. At the same time, ER-MP23⁺ and MOMA-1⁺ macrophages (M ϕ), normally present in the exocrine pancreas, migrate to the periphery of the islets (5). Surprisingly, in lymphocyte-deficient NOD^{scid} mice, the same antigen-presenting cell (APC) infiltration takes place, albeit with fewer cells (6). In NOD mice, subsequent T-cell migration to the pancreas and home around the islets (peri-insulinitis) is followed by APC and T-cell infiltration into the islets (insulinitis) (7). Destructive insulinitis coincides with BM8⁺ M ϕ infiltration and finally leads to β -cell destruction and the resulting symptoms of type I diabetes (5-7).

Until now, we and others have described several abnormalities of the islets of Langerhans and surrounding tissue in prediabetic NOD mice compared to one or several control strains. These abnormalities are summarized in Figure 1 and consist of: at birth - increased β -cell apoptosis (8), β -cell hyperactivity (*in situ* hybridization) associated with higher numbers of immature islets, reflecting islet neogenesis (9), and mild infiltration

Figure 1 - Kinetics of abnormalities observed at the pancreas level in prediabetic female NOD mice. APC: Antigen-presenting cells; ECM: extracellular matrix; GABA: gamma-aminobutyric acid; IHC: immunohistochemistry.



with DC and M ϕ (10); at 4-8 weeks of age - high percentages of adult β -stem cells (SOM+/PDX1+) (11), hyperinsulinemia (12), hyperglucagonemia (9), and progressive disappearance of GABAergic innervation (13); >10 weeks of age - mega-islet formation accompanied by extensive infiltration with DC/M ϕ and lymphocytes predominantly around these mega-islets (6). Finally, after 12 weeks of age, these prediabetic abnormalities are followed by the appearance of the first clinical signs of β -cell destruction, i.e., onset of clinical diabetes (1,2).

It is worth noting that some of these pancreatic abnormalities - including mild DC/M ϕ infiltration - are present at birth and also in NOD mice bearing the *scid* mutation which lack functional lymphocytes (1,6). Therefore, these anomalies 1) develop during fetal life, 2) are inherent to the NOD background, and 3) are not induced by lymphocytes. Moreover, the early mild perinatal, and later and stronger postweaning DC/M ϕ influxes have always been associated with abnormal islet differentiation and function. Finally, the localization of the early immune cell infiltration (APC and lymphocytes) was intriguing: around ducts and especially islets, where the targets of the autoimmune reaction, i.e., the β cells, form the core of the islet and are surrounded by non- β cells. More particularly, we frequently found APC surrounding small islets connected to ducts (Figure 2), which correspond to islets undergoing neogenesis (14,15). However, some types of these APC were also similarly localized in early postnatal pancreata from normal mouse strains (10). Therefore, these observations led us to consider the possibility of an abnormal pancreas development in mice with the NOD genetic background.

A role for APC in pancreas development?

The pancreas arises from an endodermal

budding of the embryonic foregut into the surrounding mesenchyme (15). During branching, differentiation, morphogenesis and growth of the pancreas, mesenchymal-epithelial cell interactions are important (16). Both endocrine and exocrine tissues are thought to arise from ductal epithelial stem cells through differential activation (17,18). The pancreas reaches its adult size after weaning, due to the marked postnatal growth of pancreatic tissue, especially the exocrine compartment (14). Accordingly, in the pancreas of normal rodents, particularly until 4/5 weeks of age in mice, tissue remodeling phenomena exist, consisting of waves of neogenesis, cell replication and apoptosis (19,20). A variety of cytokines and growth factors (such as IL-6, TNF- α , IFN- γ , TGF- β , HGF, EGF, NGF, VEGF, IGFI and II) (21), cell-adhesion molecules (cadherins, neural cell adhesion molecule) (22) and extracellular matrix proteins (ECM) and metalloproteinases have been reported to be involved in

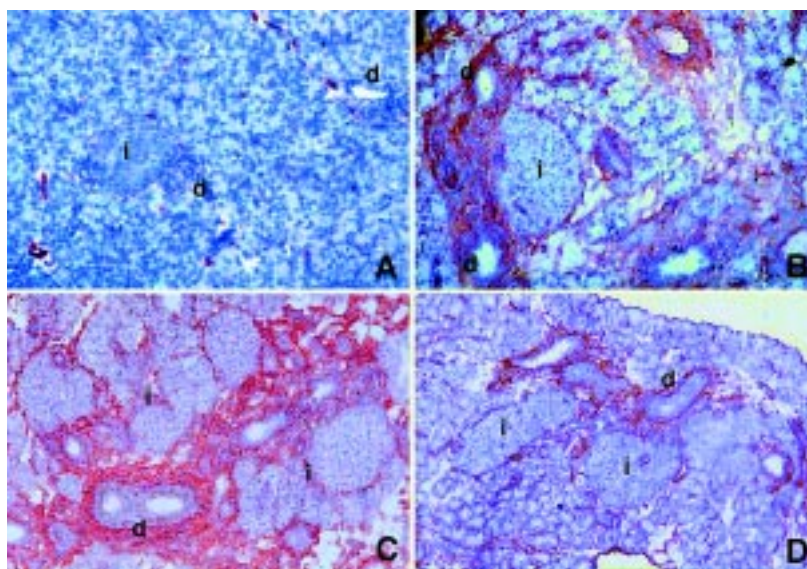


Figure 2 - A, Scavenger BM8⁺ macrophages are present in the pancreas of control strains, at 2 weeks of age, and are scattered throughout the exocrine tissue and/or are in close contact to ducts (d) and islets (i) (320X). These cells are also observed in similar localizations in NOD mice; B, C, and D correspond to fibronectin (320X), collagen I (320X) and TR-7 (fibroblast-like cells) (200X), respectively, in the pancreata of one-week-old NOD mice. These molecules are labeled in perivascular, periductular and peri-islet localizations, as for BM8⁺ macrophages. Control strains show usually thinner labeling in the same localizations (data not shown).

pancreas development (17,23).

Although DC/M ϕ are potentially able to produce and/or degrade some of these factors, nothing is known about the role that these cells may have in mesenchymal-epithelial interactions, branching and islet and/or acinar morphogenesis during pancreas development. This role could involve, for example, synthesis and degradation of ECM proteins, which are known to be controlled by metalloproteinases, synthesis of cytokines and growth factors, and apoptosis induction (24). Notably, APC and lymphocyte infiltrations have been seen during normal fetal pancreatic development in humans (25) and high numbers of APC were also observed in rat foregut during normal development (26). When we investigated the presence of different types of APC during early postnatal development in NOD strains and various control strains, we found in the latter at birth high numbers of macrophages, particularly scavenger BM8⁺ cells, scattered or in periductular and peri-islet areas, as previously described (10). However, their numbers subsided gradually and BM8⁺ cells disappeared completely during the postweaning period, in contrast to what happened in NOD mice. These data led us to hypothesize that macrophages are indeed involved in normal postnatal growth and differentiation. Our hypothesis has recently been strengthened by the observation that M ϕ , in association with eosinophils, are involved in normal postnatal mouse mammary gland development, by regulating branching morphogenesis (27).

Is ECM protein involvement abnormal during postnatal NOD pancreas development?

As regards the normal rodent pancreas, a few reports have shown *in vitro* that ECM proteins affect fetal or neonatal endocrine tissue development: laminin induces β -cell differentiation in mouse fetuses (28), fibronectin stimulates islet proliferation and insu-

lin secretion in newborn rats (29), and collagen affects the islet distribution of β versus non- β cells (30). Moreover, the levels of some ECM-degrading metalloproteinases are able to affect *in vitro* rat islet morphogenesis (17). Recently, laminin and its receptor, the $\alpha 6$ -containing integrin, have also been shown to play roles *in vitro* in ductal morphogenesis and the induction of exocrine tissue, respectively (31). *In vivo*, the expression of various ECM proteins has been evaluated during pancreas development in normal rats (23) and in TGF- $\beta 1$ -transgenic mice (32). In the latter study, increased fibroblastic proliferation and accumulations of laminin and fibronectin were observed concomitant with decreased acinar proliferation together with macrophage and neutrophil infiltration. In this regard, the haptotactic properties of ECM proteins (mainly produced by fibroblasts) acting on leukocytes are well known (33).

To the best of our knowledge, ECM protein expression has not been assessed systematically during normal postnatal mouse development. Moreover, taking into account the various abnormalities present at birth in the NOD mouse, particularly the APC infiltration, we raised the hypothesis of an ECM-related dysfunction during pancreas development in the NOD mouse. We therefore evaluated the expression of laminin and fibronectin and their corresponding integrin receptors (VLA6 for laminin, VLA4 and VLA5 for fibronectin) in the postnatal developing NOD pancreas, compared with various control mouse strains (Homo-Delarche F, Pleau JM, Durant S, Alves V, Coulaud J and Savino W, unpublished results). Preliminary data were also obtained for collagen I.

First, all molecules studied are present in mouse pancreata from birth onwards, as assessed by immunohistochemistry, and their mRNA expression was confirmed by the reverse transcriptase-polymerase chain reaction (RT-PCR). Second, various structures (for example, belonging to exocrine, endo-

crine and connective tissues, and vessels and nerves) may be labeled by a given antibody. Third, the main conclusion is that, in all strains at birth, immaturity is found at the level of various pancreatic structures: endocrine, exocrine, vascular and neural, and this was most clearly demonstrated by VLA6 labeling. Moreover, at birth, this labeling highlighted wide connective septa in NOD and NOD*scid* strains but they were labeled apparently in a nonspecific way by goat anti-mouse IgG and rabbit anti-rat IgG only in NOD mice and not NOD*scid* mice (Homo-Delarche F, unpublished data). Such labeling in NOD mice might be reminiscent of matrix alterations (matricryptic sites), occurring during tissue injury and providing important new signals to regulate the repair process (34). Fourth, some ECM proteins are well represented not only in perivascular and periductular areas but also at the ductular-vascular pole of the islet of Langerhans, as illustrated in Figure 2B and C, for fibronectin and collagen I. It should be emphasized that these localizations, and particularly the ductular-vascular pole of islets in neoformation, are also those where DC/M ϕ are found, both in control and NOD strains during the early postnatal period. Fifth, in NOD mice around weaning, laminin- and fibronectin-labeled vessels and ducts appear larger, as do vessels immunoreacted for VLA5.

Since fibroblasts may produce these ECM molecules, we used the anti-TR-7 monoclonal antibody (TR-7mAb) which has been reported to recognize mouse fibroblasts (35). TR-7mAb labeling was observed at the same sites as ECM proteins (perivascular, periductular and peri-islet areas and vascular-ductular poles of islets; Figure 2D). Moreover, in one-month-old NOD mice, TR-7mAb labeling appeared denser in these areas and, curiously, in periacinar areas. In the latter localization, these cells might correspond to the periacinar fibroblast-like cells, which stimulate acinar cell proliferation and regeneration, and are involved in periacinar fibro-

sis (36). Finally, the presence of TR-7⁺ fibroblast-like cells and ECM fibers coincides with that of primary infiltrating immune cells. However, it is worth noting that only a few macrophages (but not yet lymphocytes) are observed when many TR-7⁺ cells are already present.

In summary, this study on ECM proteins and their receptors clearly shows, for the first time, the immaturity of the mouse pancreas at birth, even in control strains. Moreover, postnatal NOD pancreas development exhibits several differences. Normally, immaturity also exists at birth at the salivary gland level, another foregut-derived organ (37) and target for the autoimmune reaction in NOD mice. Recently, NOD salivary glands were shown to undergo abnormal organogenesis, consisting of delayed morphological differentiation with wide connective septa, less acinar proliferation at birth, elevated activity of matrix metalloproteinase 9 along with collagen IV and increased expression of FasL, Fas and Bcl-2 (38). Taken together, these NOD mouse abnormalities might be a consequence of disturbed fetal foregut development. They also suggest that a more common pathophysiological mechanism than previously thought may underlie the progression of the autoimmune reaction at different organ levels in NOD mice but its clinical expression depends only on the target tissue itself. In this regard, it has already been suggested that organs derived from the same embryonal germ layer express common germ layer-specific antigens that could serve as target antigens for the autoimmune response (39).

Other pertinent conclusions of this study are: 1) islet neogenesis involves several ECM molecules and their receptors, in particular at the ductular-insular pole and its periphery, where we previously demonstrated the presence of various types of APC; 2) TR-7⁺ fibroblast-like cells, as potential producers of ECM proteins, are also an abundant component in various areas and particularly at

the islet periphery and ductular-insular pole.

These data highlight the extent of remodeling phenomena taking place in the early postnatal pancreas, in which fibroblasts and APC may play important roles even during normal development. Moreover, in NOD mice around weaning, vessels, ducts and islets in neof ormation are clearly different in terms of size, as shown by the expression of ECM molecules and their receptors and TR-7⁺ cells. The question therefore arises whether these remodeling phenomena are well controlled in the NOD mouse and, if not, at what level they are defective and to what extent they could be responsible for the autoimmune reaction.

Are apoptosis and anti-apoptosis molecules abnormally expressed during postnatal NOD pancreas development?

Since apoptotic phenomena are a normal component of development (19,40), we searched for the expression of apoptosis- and anti-apoptosis-related molecules (FasL, Fas and Bcl-2, as already done in salivary

glands) in the pancreata of various control strains and NOD and NOD^{scid} mice, using immunohistochemistry and RT-PCR (Durant S, von Bockland S, Pleau JM, Coulaud J, Alves V, Versnel M and Homo-Delarche F, unpublished results).

The most impressive data concern the presence of high numbers of FasL⁺ cells in mice with the NOD genetic background at birth. The numbers of FasL⁺ cells in NOD strains decrease slightly with time during the first month of life, while in control strains, they are scarcely present regardless of the age studied. These cells are either scattered or, once again, are found in perivascular, periductular and peri-insular localizations and at the ductular-insular pole of the islet, as are APC and TR-7⁺ fibroblast-like cells. The phenotype of these FasL⁺ cells has not yet been determined. It is worth noting that, while APC and fibroblast-like cells were always present in control strain pancreata during the postnatal period, the presence of high numbers of FasL⁺ cells in the same localizations was specific to the NOD genetic background.

Because of the different infiltration patterns of FasL⁺ cells, we wondered whether apoptosis differed among the various strains. We therefore assessed the numbers of apoptotic cells using the TdT-mediated dUTP-X nick end labeling (TUNEL) method. We chose to count all pancreatic apoptotic cells/mm², regardless of their type, taking into account the extra-insular distribution of FasL⁺ cells. We also searched for qualitative differences in Fas/Bcl-2 expression by target cells.

Figure 3 summarizes the kinetics of such determinant. First, the total numbers of pancreatic apoptotic cells do not appear to differ among strains, including NOD, but they appear to decrease gradually over the first month of life. However, by assessing only islet-cell apoptosis, other investigators found increased β -cell apoptosis in NOD mice from birth onwards (8). Second, as mentioned above,

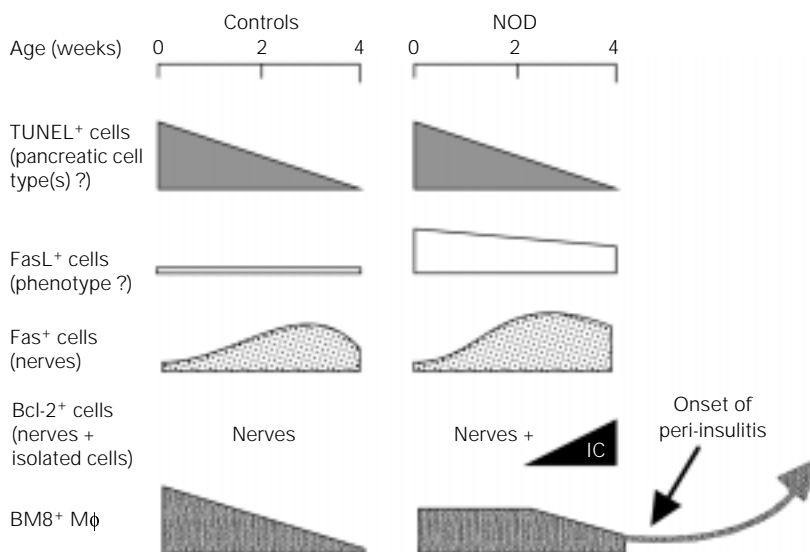


Figure 3 - Evolution of apoptosis (anti-apoptosis)-related phenomena and pancreatic BM8⁺ macrophage infiltration during the postnatal period in non-obese diabetic (NOD) mice. IC: Isolated cells; TUNEL: TdT-mediated dUTP-X nick end labeling.

there are many FasL⁺ cells in mice with the NOD genetic background from birth onwards and they do not disappear with time. Third, still only in NOD strains, a wave of isolated Bcl-2⁺ cells is observed at around 2 weeks of age and concomitantly, fibroblast-like cell infiltration appears to be denser. Fourth, when apoptotic cells are present during postnatal pancreas development, BM8⁺ scavenger M ϕ are also observed, but their evolution pattern is quite different in NOD mice compared to controls, perhaps due to the presence of FasL⁺ and Bcl-2⁺ cells. Finally and curiously, Fas⁺ and Bcl-2⁺ innervation is present during postnatal pancreas development in all strains.

Taken together, these data for NOD mice suggest a perturbation of apoptotic phenomena that takes place during postnatal pancreas development in which many actors may participate before any obvious lymphocyte infiltration. Moreover, these disturbances of pancreas development appear to be strongly dependent on the NOD genetic background because they are also observed, to some extent, in lymphocyte-deficient NODscid mice.

Conclusion: Do studies on postnatal NOD pancreas development offer a new scenario for the pathogenesis of type I diabetes?

Our studies led us to hypothesize that normal mouse pancreas development, based on epithelial-mesenchymal interaction, is characterized by a transient inflammatory process that takes place during the postnatal period. Underlying inflammation has recently been proposed as an event during organogenesis of the lymphopoietic-hematopoietic system, based on hematopoietic cell-mesenchymal cell interactions (41). In the pancreas, all kinds of partners exist (Figure 4): epithelial cells forming ducts and ductules, among which are the precursors of endocrine and exocrine tissues; mesenchymal cells, represented by fibroblasts, and he-

matopoietic cells, mainly represented by different types of M ϕ and a few DC. Moreover, innervation appears to be a widespread component of the developing pancreas. Some of these structures possibly represent the so-called "neuro-insular" complexes which were described many years ago and disappear with age (42). Their presence is intriguing, in light of their localization at the ductular-vascular pole of the islet and at its periphery in contact with the early infiltrating APC, their labeling by Fas and Bcl-2, and the fact that various molecules are common to neural and β cells and antibodies to them are found in type I diabetes (1).

If any or many of the cell type(s) involved in pancreas development do not function normally, the underlying inflammatory process may go awry. As mentioned above, hematopoietic cells such as M ϕ and eosinophils participate in ductal branching during postnatal mouse mammary gland development (27); such a mechanism may also occur in the pancreas. Moreover, M ϕ are able to produce many factors (cytokines, growth factors, metalloproteinases) potentially involved

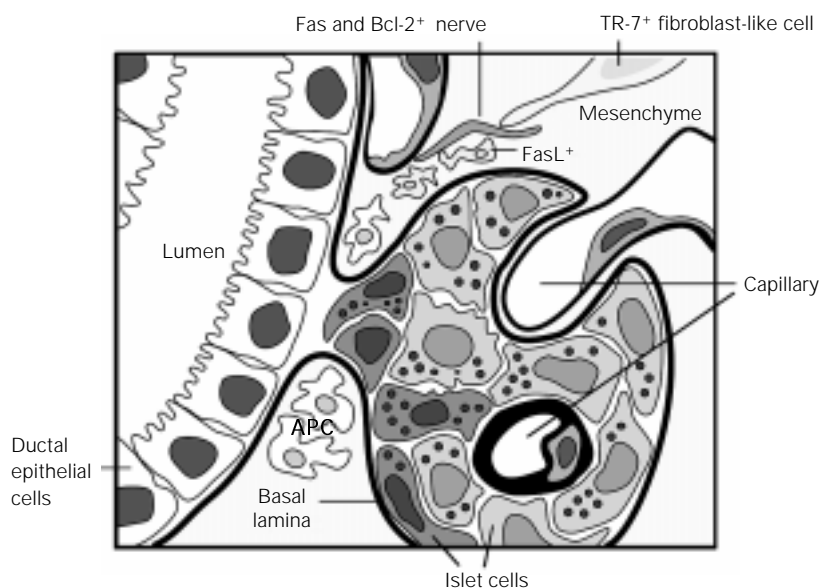


Figure 4 - Antigen-presenting cells (APC) as candidates for tissue remodeling phenomena (ECM synthesis/degradation, apoptosis) during islet neogenesis. Adapted from Pictet and Rutter (14).

in pancreas development, and particularly islet differentiation (21). Their localization around ducts, islets and at the ductular-vascular pole of the islet makes them good candidates for roles in remodeling phenomena *in vivo*, comprising islet neogenesis. Several genetic and functional defects have been described at the APC level in NOD mice, including abnormal differentiation and function, such as antigen presentation, decreased cytokine secretion and enhanced arachidonic acid metabolism, and defective Fc γ RII gene expression (43-48). Their resulting inefficacy might explain the aberrant presence of FasL⁺, Bcl-2⁺ and TR-7⁺ cells in NOD mice in the same localizations as APC.

Until now, while the role of the mesenchyme has been well recognized in *in vitro* pancreas development, the localization of fibroblasts has been overlooked *in vivo*. However, these cells are among the most common in tissues, participate in the production of ECM proteins and are actively engaged in the recovery from tissue damage and inflammation (49). During early postnatal pancreas development, we systematically found fibroblasts together with the ECM molecules they synthesize at the periphery of islets undergoing neogenesis and the ducts from which they derive. Recently, these cells, like "sentinel" cells, have been shown to synthesize chemokines that appear to be an important link between innate and adaptive immunity (49). Moreover, the ECM serves as a specialized reservoir for cytokines and growth factors that promote cell proliferation, differentiation, activation, migration and apoptosis (24,50). The ECM microenvironment also possesses a myriad of signals that dialogue with immune cells, coordinating their behavior as they make their way into inflamed tissue (50). It is presently not known whether fibroblasts are able to communicate normally with APC and/or ductal epithelial cells during NOD pancreas development. However, it has already been suggested that the diabetic defect(s) can arise from extrapancreatic cells of mesenchymal

origin (51).

It is not clear whether lymphocytes may also participate in normal pancreas development; however, their presence has been described in human fetal pancreata (25,52). Pertinently, this infiltration consists of large focal infiltrates of T cells and DC/M ϕ , localized in the connective tissue of septa and capsule of the pancreas. Later, from birth to around weaning, they might be involved in peripheral tolerance as already described for skin epithelium (53). In NOD mice, the potential anomalies at lymphocyte levels include deficient central tolerance (54), which favors autoimmunity, and resistance to apoptosis (55,56), which can lead to the *in situ* accumulation of autoreactive and naive lymphocytes. Moreover, Fas/FasL-induced apoptosis is also critical for tolerance induction, and its dysregulation may also trigger autoimmune processes (57).

Finally, the main notion that can be derived from these findings is that type I diabetes might be a disease of pancreas development and particularly of islet neogenesis. This possibility could reasonably explain why type I diabetes 1) is principally a disease of childhood; 2) is affected by various gestational events, and 3) emerges when glucose homeostasis is perturbed with increased insulin demand (puberty, pregnancy, infections, stressful events) (1,3). The hypothesis of altered pancreas development in type I diabetes may lead to a better understanding of 1) the multiplicity of the potential autoantigens; 2) the nonspecificity of islet cell antibodies (ICA) and islet cell surface antibodies, recognizing islet cells other than β cells; 3) the presence of autoantibodies recognizing molecules common to β cells and nerves, such as glutamic acid decarboxylase (GAD), and 4) the presence, in the fetal pancreas, of some type I diabetes-associated antigens (GAD65, ICA69) in epithelial and/or ductal cells (1,58,59).

But how can we explain the apparent β -cell specificity of the disease? First, β cells

play a crucial role in the organism. Second, hyperactive β cells are more susceptible to aggression: hyperactive endocrine cells are more prone to autoimmune reactions because of higher surface expression of autoantigens and adhesion and MHC molecules, and because of an enhanced sensitivity to cytokine-induced damage (4). In the case of increased insulin demand and hyperactivity, there is a fine balance between islet neogenesis and β -cell apoptosis (60). In NOD neonates, β -cell hyperactivity together with islet neogenesis and β -cell apoptosis, is already present, thereby exposing potential autoantigens (8,9). The different waves of NOD β -cell hyperactivity appear to be triggered by maternal abnormalities of glucose homeostasis and normal dietary changes, which take place during the weaning period. Later, during the progression of the autoimmune reaction, and because of a defective islet neogenesis attributable to immune cell abnormalities, the exhausted and finally dying β cells would not be replaced, leading to the clinical onset of the disease.

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