Leukocyte Extravasation into the Pancreatic Tissue in Transgenic Mice Expressing Interleukin 10 in the Islets of Langerhans

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Summary

Transgenic expression of interleukin 10 (IL-10) in the islets of Langerhans leads to a pronounced pancreatic inflammation, without inflammation of the islets of Langerhans and without diabetes. A scattered infiltration of macrophages (Mφ) precedes localized accumulations of CD4+ and CD8+ T lymphocytes, B lymphocytes, and Mφ. This recruitment of inflammatory cells to the pancreas is somewhat surprising, since the biological activities of IL-10 in vitro indicate that IL-10 is a powerful immunosuppressive cytokine. Since endothelial cells play a major role in leukocyte extravasation, we examined if vascular changes and extralymphoid induction of peripheral and mucosal type vascular addressins contributed to IL-10-induced homing of mononuclear cells to the pancreas. The endothelium lining small vessels was highly activated in areas of inflammation, as the endothelial cells became cuboidal, and exhibited increased expression of major histocompatibility complex class II (Ia), intercellular adhesion molecule 1, and von Willebrand Factor. Furthermore, induction of vascular addressins simultaneously with accumulation of mononuclear cells around islets and vessels indicated that the endothelial cells take on the phenotype of differentiated endothelium specialized for leukocyte extravasation. In conclusion, pancreatic inflammation and vascular changes are prominent in IL-10 transgenic mice. We hypothesize that IL-10, in addition to its immuno-inhibitory properties, is a potent recruitment signal for leukocyte migration in vivo. These effects are relevant for in vivo therapeutic applications of IL-10.

IL-10 is produced from Th lymphocytes, Ly1+ B lymphocytes, and macrophages (Mφ)1 (1-3). IL-10 decreases Mφ production of IL-1, IL-6, TNF-α, and nitrogene oxide, and inhibits IFN-γ and IL-3 secretion from Th1 lymphocytes (1, 4-6). IL-10 has not only antiinflammatory properties, but stimulates differentiation of mast cells and CTLs (1). Taken together, these in vitro studies indicate that IL-10 is a powerful immunosuppressor and, therefore, may be a potential immunosuppressive compound for treatment of autoimmune diseases and allograft rejections.

To correlate in vitro observations with in vivo effects, we created transgenic mice with localized expression of IL-10. Since transgenic expression of IL-10 in pancreatic β cells led to homing of inflammatory cells into the exocrine pancreatic tissue, the expression of endothelial cell (EC) antigens, antigens specific for ECs specialized for lymphocyte diapedesis, and the expression of adhesion molecules on infiltrating cells were examined.

Materials and Methods

Construction of the Transgene. A recombinant plasmid containing the human insulin promoter and a terminator sequence from the hepatitis B virus gene (7) was cleaved at its unique BamHI site, followed by blunt-ending of the site with the Klenow fragment of DNA polymerase I. The 594-bp SacI fragment encoding the murine IL-10 was excised from a cDNA clone (8), blunt-ended as above, and inserted into the BamHI site of the insulin promoter—containing plasmid. Isolation, purification, and injection of a restriction fragment of the hybrid DNA molecule were performed as described previously (7).

Screening of Transgenic Mice. For Southern blot analysis, 5-10 μg of DNA, extracted from 2-cm-long tail segments, was digested with PstI overnight. The DNA was subjected to electrophoresis on 0.8% agarose gels and transferred to nitrocellulose membranes (NitroPure; Micron Separations Inc., Westboro, MA). Hybridization was performed with radiolabeled restriction fragments of the IL-10 cDNA (Prime-It™ Random Primer Labeling Kit; Stratagene, La Jolla, CA). Transgenic founders were bred with BALB/c mice and killed for histological characterization after transmission to one or two litters, as tested by PCR of tail DNA using transgene-specific primers. Mice were maintained in microisolator cages under pathogen-free conditions.

Expression Analysis by In Situ Hybridization. Two 3-mo-old IL-
10 transgenic mice and one normal BALB/c mouse were killed. Excised pancreata were fixed in 4% paraformaldehyde in PBS for 10 min, washed in 10, 15, and 20% sucrose, and frozen in Tissue-Tek (Miles Laboratories Inc., Elkhart, IN). The RNA probe was prepared by in vitro transcription of a linearized plasmid containing IL-10 cDNA, using digoxigenin-modified UTP (Boehringer Mannheim Biochemicals, Indianapolis, IN). In situ hybridization using nonisotype-labeled probes was performed as described (9). After overnight incubation at 37°C with 50 µl of probe-containing hybridization mixture, sections were sequentially reacted with sheep anti-digoxigenin antibody (1 µg/ml) (Boehringer Mannheim Biochemicals), followed by incubation with biotin-labeled rabbit anti-sheep IgG (7.5 µg/ml) (Vector Laboratories, Inc., Burlingame, CA), and horseradish peroxidase (HRP)-labeled avidin-biotin complex (ABC-kit; Vector Laboratories, Inc.). HRP was visualized using 3,3′ diaminobenzidine as chromogene.

**Immunohistochemistry.** 1- (n = 4), 4- (n = 3), and 6- (n = 2)-wk-old mice, and 2- (n = 1), 3.5- (n = 1), 5- (n = 2), and 6- (n = 1) mo-old mice were killed. Mice were obtained from two different lines of IL-10 transgenic mice. Normal BALB/c mice or negative littersmates served as controls. Fresh frozen sections were dehydrated in acetone for 5 min at −20°C and blocked in 2.0% bovine albumin (Sigma Chemical Co., St. Louis, MO) following the above protocol.

**Results**

**Initial Characterization of IL-10 Transgenic Mice.** Histological examination of the transgenic mice demonstrated substantial accumulations of inflammatory cells within the exocrine pancreatic tissue in five of nine independent lines of transgenic mice. The infiltrates surrounded the islets of Langerhans, ranging from a modest peri-islet infiltration in young mice (Fig. 1 A) to a profound inflammation of the exocrine tissue, in which the acini were replaced by dense sheets of inflammatory cells, necrotic tissue, and areas of adipose tissue in older mice (Fig. 1 B). Although the islets of Langerhans were embedded in a sea of leukocytes and necrotic exocrine tissue, they remained circumscribed and entirely intact, and the mice never became diabetic. Furthermore, the organization of the endocrine cells showed the normal mantle of glucagon- and somatostatin-containing cells surrounding a core of insulin-containing β cells (data not shown). The islet-specific expression of the transgene was demonstrated by in situ hybridization (Fig. 2). No hybridization was found in the pancreas of control mice.

Investigation of the chronology of the pancreatic inflammation indicated that mononuclear cells began to infiltrate the pancreas of 1-wk-old transgenic mice. At this age the majority of the infiltrating cells were identified as Mφ by the F4/80 antibody. Focal accumulations of mononuclear cells around vessels and islets were demonstrated at 4–6 wk of age, increasing at 2–3 mo of age. At 2 mo of age lymphocytes dominated the inflammation, whereas ~50% of the infiltrating cells were identified as macrophages. Of the infiltrating lymphocytes, ~40 and 30% were CD4+ and CD8+ T lymphocytes, respectively, and ~30% were B lymphocytes (Fig. 3, A–C). A subpopulation of the lymphocytes expressed the Ly1 antigen. The proportion of different lymphocyte subsets was similar at 6 mo of age.

As evaluated by bromodeoxyuridine incorporation in vivo, the mitotic activity of the extravasated lymphocytes was very low (D. Gu, unpublished observation), implying that IL-10-induced homing of leukocytes, rather than proliferation of inflammatory cells within the pancreas, was responsible for the observed lesions. Since the vessels were very prominent in the pancreatic tissue of IL-10 transgenic mice, and since leukocyte extravasation is controlled by a specific interaction between leukocyte adhesion molecules and the endothelium lining the vascular wall, we looked for the presence of endothelial changes, the possible expression of vascular addressins, and the expression of different adhesion molecules in the pancreatic inflammation.

**Characterization of the Endothelium in IL-10 Transgenic Mice.** The antibody MECA 32 recognizes an antigen present on all types of endothelium, a 60 kDa transmembrane glycoprotein of unspecified function, more commonly known as vascular cell adhesion molecule-1 (VCAM-1) or α4 integrin. As evaluated by in situ hybridization using digoxigenin-modified UTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), and examined by immunohistochemistry, MECA 32-reactive vessels were plump and cuboidal, and demonstrated increased endothelial permeability (Fig. 4, A and B). In older transgenic mice (>6–8 wk) structural changes in small vessels were demonstrated in areas of inflammation only (Fig. 4 C). ECs lining the small vessels were plump and cuboidal, and demonstrated increased staining intensity (Fig. 4 D) when compared with the ECs lining small vessels in noninflammatory areas of the same pancreas (Fig. 4 E) or with negative littersmates.

Unstimulated endothelium has low expression of intercellular adhesion molecule 1 (ICAM-1) and MHC class II antigens (11). However, the expression of ICAM-1 and Ia increases in inflammatory foci. Furthermore, exposure to...
cytokines potentiates histamine- and thrombin-stimulated secretion of von Willebrand Factor (vWF), and increased secretion and leakage of vWF into adjacent tissue is demonstrated in inflamed foci (12, 13). Pancreatic tissue from the transgenic mice was subjected to antibodies against ICAM-1, Ia, and vWF to determine if the pancreatic endothelium in the transgenic mice showed any functional changes. In pancreata of 1-wk-old transgenic mice, vessels expressing ICAM-1 and Ia appeared more frequently than in nontransgenic littermates (Fig. 5, A and B). Expression of ICAM-1 and Ia increased progressively on the surface of the ECs lining small arteries, venules, pancreatic ducts, and interacinar capillaries in mice from 4 wk to 2 mo of age (Fig. 5, C and D). Cells expressing ICAM-1 and Ia were also demonstrated in the islets of Langerhans. However, expression seemed to be localized to the capillaries and not to the endocrine cells. In pancreata from 1-wk-old normal mice only a few vessels showed expression of vWF (Fig. 6 A), whereas small vWF-expressing vessels appeared throughout the pancreas in age-matched transgenic mice (Fig. 6 B). In pancreatic tissue from normal adult mice, vWF staining had a granular appearance and was expressed on the luminal edge of scattered larger arteries and venules (Fig. 6 C). As described for rats (14), some islets of Langerhans could easily be identified by a characteristic expression of vWF (Fig. 6 C). Distant from inflammatory foci, the expression of vWF in pancreata from transgenic mice >4 wk of age was similar to normal controls, whereas small, vWF-positive vessels appeared in the infiltrates, including small vessels lined by high ECs (Fig. 6 D). Expression of vWF was induced on interacinar capillaries diverging out from inflammatory foci, and vWF was seen leaking out into the surrounding parenchyma. Pancreatic ducts embedded in inflammatory cells did not express vWF (Fig. 6 D).

During stimulation of ECs with cytokines the vascular permeability increases (15). Therefore, we tested the permeability for albumin of the pancreatic vessels. After injection of BSA, reaction of the pancreatic tissue with an albumin antibody demonstrated extravascular accumulations of albumin in inflammatory loci only (Fig. 6 E). No albumin deposits were detectable in the pancreatic tissue from untreated transgenic mice. We could not demonstrate sediments of albumin in the pancreas of a normal BALB/c mouse after injection of BSA.

Figure 1. Hematoxylin and eosin staining of pancreatic tissue from mice expressing IL-10 in the pancreatic β cells. In early stages of inflammation infiltrating cells have a peri-vascular or peri-islet localization (A) (>×80). In later stages a profound inflammation involves most of the pancreatic tissue, leaving the islets of Langerhans intact (B) (>×40). (b) Islet of Langerhans.

Figure 2. Demonstration of IL-10 expression in islets of Langerhans by in situ hybridization. Hybridization with digoxigenin-labeled probe shows islet-specific expression of IL-10.
In summary, our observations indicate that ECs are highly activated, and have increased adhesion properties. Furthermore, the increased permeability may contribute to spreading of the inflammatory process.

Expression of Vascular Addressins in IL-10 Transgenic Mice. Leukocytes may not only pass through stimulated endothelium but also through tissue-specific postcapillary high endothelial venules (HEV), specialized for lymphocyte diapedesis (16). The two best characterized types of HEV are HEV in peripheral LN, identified by the mAb MECA 79 (17) recognizing the peripheral LN vascular addressin (PNAd) (18), and HEV in mucosal lymphoid tissue (Peyer's patches and appendix), defined by the mAb MECA 367 (19) recognizing the mucosal vascular addressin (MAd) (20). Expression of peripheral and mucosal HEV in nonlymphoid organs is described in areas of chronic inflammation, contributing to homing of inflammatory cells in peripheral tissues (21–23). To examine if induction of extralymphoid HEV could be a factor responsible for homing and extravasation of inflammatory cells to the pancreas in IL-10 transgenic mice, pancreatic tissue was reacted with the antibodies MECA 367 and MECA 79. The antigen specific for HEV in Peyer's Patches, defined by MECA 367, was expressed in a scattered manner both in 1-wk-old normal and transgenic mice. Small vessels reactive with the MECA 367 antibody were induced in areas of inflammation (Fig. 7 A), even when only very few inflammatory cells were accumulating around vessels and islets. The number of small vessels expressing this antigen increased with the severity of the surrounding inflammation. The antigen specific for HEV in peripheral lymph nodes, identified by MECA 79, was not expressed in pancreatic tissue from young or adult normal mice. This antigen was expressed in small vessels in the inflammatory infiltrates in transgenic mice (>3.5 mo of age only (Fig. 7 B). The findings indicate that leukocyte extravasation in the present model of pancreatic inflammation may take place not only through activated ECs, but also through differentiated ECs with phenotypical characteristics of mucosal HEV and peripheral type HEV.

Expression of Leukocyte Adhesion Molecules in IL-10 Transgenic Mice. Since the ligand for t-selectin is expressed on peripheral LN HEV (24) and on activated endothelium (25), we tested the expression of this leukocyte adhesion molecule on the infiltrating cells. In mice >4 wk of age t-selectin-expressing mononuclear cells were demonstrated around vessels and islets of Langerhans (Fig. 8 A). The presence of t-selectin-expressing leukocytes suggests that an interaction between these cells and ligands on induced peripheral-type HEV may take place.

Because the integrin ICAM-1 is hyperexpressed in our transgenic mice, and the ECs express antigens characteristic for mucosal HEV, we tested if infiltrating cells expressed the respective integrin ligands, such as Mac-1/LFA-1 and LPAM-1/LPAM-2, respectively (10). At 1 and 4 wk of age, the diffusely infiltrating F4/80 cells predictably expressed Mac-1 and LFA-1, although the latter in smaller amounts (Fig. 8, B and C). Also, at 4–6 wk of age, when localized accumulation of F4/80 cells was evident, the cells expressed Mac-1 and LFA-1 (Fig. 8, D and E). Approximately 30–40% of
Figure 4. Pancreatic expression of a pan-endothelial marker, as defined by the antibody MECA 32. Pancreatic tissue from a 1-wk-old nontransgenic mouse (A) and from an age-matched littermate expressing IL-10 in islets of Langerhans (B) (both ×160). (C) Pancreatic tissue from an 8-wk-old IL-10 transgenic mouse demonstrating an increased number of small vessels expressing the EC marker in areas of inflammation (arrowheads) (×80). ECs in small vessels in inflammatory foci become thick and cuboidal (D) as compared with ECs in noninflammatory foci (E) (both ×400). (a) duct; (b) islet of Langerhans; (c) vessels.
the infiltrating cells expressed the α4 integrin chain of LPAM-1 and -2 in all tested mice and followed the same pattern of infiltration as described above (data not shown). The observations support the notion that extravasation of inflammatory cells in the present model of pancreatic inflammation in part is mediated by interaction between upregulated endothelial adhesion molecules and vascular addressins, and their respective leukocytic ligands.

For the understanding of the pathogenetic mechanisms in IL-10-induced inflammation, it was interesting to test if the infiltrating leukocytes exhibited high levels of ICAM-1 expression. However, in the pancreata of 1-wk-old transgenic mice, only diffusely scattered cells were ICAM-1 positive (Fig. 5 A). In older mice some of the infiltrating cells, located in the periphery of the inflammatory foci, expressed ICAM-1 on the cell surface, although a rather large number of cells did not show detectable expression of this adhesion molecule (Fig. 5 C).

Discussion

Transgenic expression of IL-10 in the islets of Langerhans leads to a pronounced pancreatic inflammation dominated by CD4+ and CD8+ T and B lymphocytes. The islets of Langerhans remain entirely intact, and the transgenic mice do not develop diabetes. The observation contrasts, at least superficially, the reported in vitro activities of human and
murine IL-10, which indicate that IL-10 is a powerful immunosuppressor (1). The chronological appearance of leukocyte subsets and the pattern of migration in IL-10 transgenic mice is similar to what is demonstrated in several animal models of insulin dependent diabetes mellitus (IDDM) (26–28) and in transgenic mice expressing IFN-γ in the pancreatic β cells (M.-S. Lee, personal communication), although the rapidity of the process may differ. This implies the existence of a common migration pattern, irrespective of the initial lesion resulting in pancreatic infiltration. Very puzzling, and in contrast to animal models of IDDM- and IFN-γ-expressing mice (7), lymphocyte penetration into islets and subsequent β cell destruction are entirely absent in IL-10 transgenic mice. The mechanism of β cell killing in IFN-γ transgenic mice is thought to occur by IFN-γ-induced activation of quiescent autoreactive lymphocytes in vivo (29). In contrast, the absence of β cell destruction in IL-10 transgenic mice may be explained by IL-10-induced inhibition of the antigen-presenting capacity of Mφ, inhibition of antigen-specific T cell proliferation, and reduced cytokine production; consequently, the β cells are not exposed to cytotoxic cytokines and antigen sensitization is prevented. However, the lack of insulitis in the IL-10 transgenic mice could reflect differences in the expression pattern of appropriate adhesion molecules.

Figure 6. Pancreatic expression of vWF and demonstration of increased permeability. The expression pattern of vWF in pancreatic tissue from a 1-wk-old normal (A) and from a 1-wk-old IL-10 transgenic mouse (B) (both ×60). The latter shows an increase in the number of small vessels expressing vWF. vWF expression in an islet of Langerhans in an adult normal mouse (C) (×60). Demonstration of pronounced vWF staining in larger vessels, and the appearance of smaller vessels expressing vWF in inflamed loci (D)(arrowheads) (×30). For the demonstration of increased vascular permeability transgenic mice were injected intravenously with 0.5 g BSA. After 30 min the mice were killed, and the pancreases were processed for immunohistochemistry. The presence of albumin in inflamed loci indicates an increased vascular permeability (E) (×120). (a) duct; (b) islet of Langerhans; (c) vessels.
Figure 7. Pancreatic expression of vascular addressins. Expression of the mucosal (arrowheads) (A) and the peripheral LN (arrowheads) (B) vascular addressins in IL-10 transgenic mice, identified by the antibodies MECA 367 and MECA 79, respectively (both ×80). (a) duct; (b) islet of Langerhans.

on the islet vasculature, inhibiting β cell exposure to the immune system.

Since murine IL-10 enhances the growth factor–induced proliferation of mature CD4+ and CD8+ T lymphocytes (30), and enhances the differentiation of cytotoxic T cell precursors into CD8+ cytotoxic T cells (31), local proliferation of lymphocytes could in part explain the accumulation of inflammatory cells in the pancreas. However, the mitotic activity of the extravasated lymphocytes was very low. To elucidate the role of the endothelial cells in IL-10–induced leukocyte extravasation, we examined the pancreas for vascular changes. The functional and structural changes of the endothelium, such as increased expression of ICAM-1, Ia, and vWF, increased permeability, neoexpression of vascular addressins, and appearance of small vessels with cuboidal ECs, indicate that the ECs are highly activated (11-13) and take on the phenotype of differentiated endothelium specialized for leukocyte extravasation (16, 32). The leukocyte ligands for ICAM-1 (Mac-1 and LFA-1), Mad (LPAM-1/2), and PNad (1-selectin) were expressed on a majority of the infiltrating cells mediating adhesion to the activated endothelium and neo-expressed vascular addressins. Therefore, our data suggest that IL-10 is a potent signal for leukocyte extravasation in vivo.

Expression of MHC class I and II, and ICAM-1 on ECs, are influenced by cytokines at sites of inflammation (33-38). Furthermore, cytokines, particularly IFN-γ, are decisive for the maintenance and “adhesiveness” of peripheral LN and mucosal HEV (39-42). This indicates that normal “flat” endothelium may differentiate into endothelium with HEV characteristics by exposure to IFN-γ and other cytokines in inflammatory foci, e.g., in arthritis, experimental allergic encephalomyelitis (21, 23, 43), or as reported herein. Since we did not find any evidence of vasculogenesis, indicated by the absence of mitotic activity in small vessels, it is most likely that preexisting vessels develop phenotypic characteristics of HEV. In IL-10 transgenic mice the initial observation was stimulation of the ECs, indicated by increased expression of ICAM-1, Ia, and vWF, and homing of Mac-1-positive cells to the pancreas. Preliminary studies show that IL-10 induces ICAM-1 expression on ECs in vitro. Therefore, vascular changes in vivo may, at least partly, be mediated directly by IL-10, although the Th1 cell production of granulocyte-MΦ/monocyte-CSF (GM-CSF), lymphotoxin (LT), and TNF-α, which is not downregulated by IL-10 (44), and thrombin and histamine, produced from the infiltrating cells, most likely plays a major role in the differentiation of the ECs.

In conclusion, transgenic expression of IL-10 in pancreatic β cell leads to a pronounced pancreatic inflammation. Homing of target cells to the location of IL-10 production may be important for the manifestation of the immunosuppressive function on otherwise distant, circulating leukocytes. We hypothesize that IL-10, in addition to its in vitro immunoinhibitory properties, can attract leukocytes in vivo. These results may have important consequences on the therapeutic use of IL-10.
Figure 8. Expression of leukocyte adhesion molecules in IL-10 transgenic mice. To identify the presence of different leukocyte adhesion molecules, pancreatic tissue was reacted with antibodies recognizing 
9-selectin (A) (×120), Mac-1 (B and D), and LFA-1 (C and E). Mac-1-positive cells were scattered in 1-wk-old mice (B) (×120), but showed a localized distribution in older animals (D) (×120). A similar pattern was demonstrated for LFA-1 expression in young (C) (×120) and older mice (E) (×120). (b) Islet of Langerhans.

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