Journal of Cellular Biochemistry 85:123–130 (2002)
DOI 10.1002/jcb.10114

Cytokine Regulatory Effects on α-1 Proteinase Inhibitor Expression in NOD Mouse Islet Endothelial Cells

Gianpaolo Papaccio,[1]* Marcella Pedullà,[2] Eduardo Ammendola,[1] and Matilde Todaro[3]

[1]Department of Experimental Medicine, Laboratory of Histology, School of Medicine, 2nd University of Naples, 5 via L. Armanni, 80138 Naples, Italy
[2]Department of Pediatrics, School of Medicine, 2nd University of Naples, 4 via L. De Crecchio, 80138 Naples, Italy
[3]Department of Surgical and Oncological Sciences, University of Palermo, Palermo, Italy

Abstract Human microvascular islet endothelial cells (IEC) exhibit specific morphological and functional characteristics that differ from endothelia derived from other organs. One of these characteristics is the expression of α-1 proteinase inhibitor (Api). In this study, we observed its expression in nonobese diabetic (NOD) mouse IEC, in relation to the occurrence of type 1 diabetes and in response to cytokines, namely IL-1β and IL-10. In addition, IL-10-deficient NOD mice as well as IL-10 transgenic NODs were studied. Results have demonstrated that Api expression is: (i) highly specific for IEC in NOD mouse islets, as for humans; (ii) linked to the occurrence of early type 1 diabetes, and iii) strongly modulated by Th1 and Th2 cytokines. In fact, Api mRNA found in pre-diabetic NOD animals is significantly reduced when they become hyperglycemic and disappears by 25 weeks of age, when mice are diabetic. Moreover, Api mRNAs are never seen in nondiabetic controls. Furthermore, in cultured NOD IEC, Api expression is downregulated by the addition of IL-1β and is upregulated by IL-10; it is always absent in IL-10-deficient NOD mice and overexpressed in IL-10 transgenic NODs, thus further supporting that this cytokine upregulates Api expression. J. Cell. Biochem. 85: 123–130, 2002.

Key words: α-1 proteinase inhibitor; islets; endothelia; cytokines; transgenic mice

It is well known that a microcirculatory system, with a particular distribution and relationship with the endocrine cells, supplies the islets of Langerhans [Bonner Weir and Orci, 1982; Samols et al., 1988; Stagner et al., 1988]. It has been demonstrated that the islet microvascular system is considerably altered during diabetes occurrence [Papaccio et al., 1990; Pober and Cotran, 1990], and that it may also play a role in the pathophysiology of diabetes [Papaccio and Chieffi Baccari, 1992; Papaccio, 1993].

In vitro studies have recently shown that purified human microvascular islet endothelial cells (IEC) express unique morphological and functional characteristics, which differ from endothelia of other organs [Lou et al., 1999]; these characteristics are closely related to the expression of α-1 proteinase antitrypsin, an important proteinase inhibitor [Lou et al., 1999]. It has been also demonstrated that this α-1 proteinase inhibitor (Api) is specifically synthesized by IEC rather than taken up from the serum [Lou et al., 1999]. Furthermore, Api has been found to be not only a proteinase inhibitor, but also an immune regulator capable of modulating lymphocyte proliferation, cytotoxicity, transendothelial migration, and monocyte and neutrophil functions. It thus exerts a role mainly in immune disorders [Breit et al., 1985] and particularly in type 1 diabetes [Sandler et al., 1987]. These reports also suggest that its expression may have an important physiological significance in islet homeostasis.

Therefore, the aim of this study was to observe the expression of α-1 antitrypsin inhibitor in microvascular IEC of nonobese diabetic (NOD) mice in relation to early diabetes occurrence and

Grant sponsor: National Italian Ministry for University (MURST); Grant sponsor: Regional Funds (Campania Region) (to G.P.); Grant number: L.R. 41/94.

*Correspondence to: Prof. Gianpaolo Papaccio, Department of Experimental Medicine, Laboratory of Histology, 2nd University of Naples, School of Medicine, 5 via L. Armanni, 80138 Naples, Italy. E-mail: gianpaolo.papaccio@unina2.it

Received 10 September 2001; Accepted 3 December 2001

in response to two cytokines, namely IL-1β and IL-10, as well as in IL-10-deficient and IL-10 transgenic NODs.

Our results demonstrate that Api is highly specific for IEC in mice, as for humans, and that its expression is highly modulated by cytokines; actually, IL-1β downregulates and IL-10 upregulates its expression. IL-10 induced-expression is substantiated by the use of IL-10-deficient NOD mice, which do not express Api, and by the further observation that IL-10 transgenic NODs express the said proteinase inhibitor.

MATERIALS AND METHODS

Animals

Female NOD mice were purchased from Bommice (Bomholtgarten, Denmark), and housed in our facility. NOD-IL-10-deficient (knock-out homozygous IL-$10^{-/-}$) mice were generated following the Balasa et al. [2000] schedule. Briefly, IL-10-deficient (−/−) C57Bl6/J mice were purchased from form the Jackson Laboratory (JAX® GEMM™ Strain, Harbor, Maine); then they were backcrossed to NOD mice (Bommice Bomholtgarten, Denmark) for 9 generations until 12 generations. Mice of N10 to N12 backcross generations were intercrossed and used in our experiments. Heterozygous mice were intercrossed to generate homozygous (−/−), heterozygous (+/−), and wild-type (+/+) mice. The primers used to type mice for IL-10 gene disruption were provided by the Jackson Laboratory, as indicated before [Balasa et al., 2000a] and used to type mice for IL-10 gene disruption. NOD-*Il10*^{tm1Cgn} transgenic mice, overexpressing IL-10, were originally obtained from the Jackson Laboratory (JAX GEMM Strain, Harbor, Maine). The breeding of these transgenic mice was achieved after determination of the mIL-10 cDNA sequence (7.2-kb segment on chromosome 1); they are homozygous and fully congenic as they have been backcrossed onto the background strain for at least ten generations. Animals had free access to tap water and standard laboratory diet and were maintained in pathogen-free conditions. They were subjected to a 12-h light/12-h dark schedule. During the experiments, they were free of viruses or other bacterial pathologies. Ten-, fifteen-, twenty-, and twenty-five-week-old NOD females (n = 6 animals per each age and per experiment) were used for the studies which were made in triplicate. Animals were decapitated under anesthesia, and pancreas was removed and processed as described below. NOD IL-10-deficient, as well as NOD-*Il10*^{tm1Cgn} (transgenic) mice of 15–20 and 25 weeks of age were also used for the same purpose (n = 6 animals per each age and experiment). C57BL6/J mice, of the same age, fed ad libitum and housed in our facility were used as nondiabetic controls (n = 5 per each age and experiment).

Glycemia

Blood glucose levels were checked 2 h before the start of the experiment and before killing, using a glucometer (Boehringer-Mannheim, Germany). Animals were considered hyperglycemic when their fasting blood glucose levels were higher than 8 mmol/L, but lower than 12 mmol/L in two successive determinations. Mice were considered diabetics when their blood glucose levels exceeded 12 mmol/L.

Standard Light Microscopy and Assessment of Insulitis Severity

Pancreatic samples from each animal were fixed with Bouin's fixative and embedded in paraffin. Specimens were sectioned serially (5 μm thick) and stained with hematoxylin-eosin or Gomori aldehyde fuchsin for general morphology and for the evaluation of islet and extra-islet infiltration. Insulitis severity was assessed by two independent researchers who scored the islets as follows: each islet was assigned a numerical value to describe insulitis severity: 0, no infiltration; 1, peri-insulitis; 2, peri-insulitis with single leukocytes invading the islet parenchyma; 3, < 50% intraislet infiltration; 4, invasive insulitis (> 50%); 5, complete infiltration (up to 100%); 6, islet atrophy and retraction due to islet β cell loss. For each section, scores were summed and divided by the total number of islets counted to obtain an average score of insulitis severity.

Ex-Vivo Immunocytochemistry

Immunocytochemical observations were made ex vivo on pancreata taken from the animals. The anti-Api antibody (Rockland immunochemicals, Gilbertsville, PA) was FITC-conjugated and used on pancreatic tissues using standard techniques.

Endothelial Cells: Outgrowth, Isolation, and Characterization

Microvascular IEC were isolated from hand-picked islets by outgrowth on a collagen type 1 matrix, as previously described [Suscheck et al., 1994]. Iodoacetic acid (4 μg/ml) was added to the medium in order to inhibit fibroblast contamination and growth. Pancreatic islets were harvested after ductal injection of collagenase type V (Sigma, Milan, Italy) and subsequently centrifuged on a Ficoll-Histopaque (Sigma) density gradient. Hand-picked whole islets, and aortic segments (rings < 2 mm in width removed from thoracic aorta, rinsed in Hanks' balanced salt solution and cleaned from fat and connective tissue), as well as large liver vessels were placed on top of the collagen gel (1.8 mg/ml) in 24-well tissue culture plates and incubated in RPMI 1640 (Sigma) with 20% fetal calf serum (FCS), 100 μg endothelial cell growth supplement/ml, and heparin (100 μg/ml-Sigma) in a humidified incubator at 37°C in a 95% air/5% CO_2 atmosphere for 5–8 days, depending on the degree of outgrowth of endothelial cells. Islet and vessel explants from thoracic aorta, lung, and liver were then removed, cells detached with 0.25% collagenase in Hanks' balanced salt solution and replaced in plastic culture dishes with RPMI 1640 supplemented with 20% (vol/vol) FCS and 100 μg/ml endothelial cell growth supplement (ECGS) (Sigma). Cells were subcultured for up to ten passages, and removal from culture dishes for each passage was performed by treatment with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid in isotonic NaCl for 3 min. In some experiments, IEC from 10- and 20-week-old NOD mice and from nondiabetic controls were cultured in the presence of murine rIL-1β (50 U/ml) or rIL-10 (50 U/ml) (Sigma), and the culture continued for 2 days. All the assays were performed both at the beginning and at the end of the culture. The above mentioned concentrations of cytokines were choosen after having done preliminary experiments in which dose–response was evaluated. At lower concentrations (20 or 30 U/ml), the cytokines did not elicite responses and higher concentrations (100–300 U/ml) were toxic for IEC; their morphological appearance was modified, and in particular, rIL-1β showed apoptotic effects at a dose of 300 U/ml.

For characterization, cells were passaged from tissue culture dishes onto sterile glass coverslips and allowed to grow as subconfluent monolayers. Cells were washed with phosphate-buffered saline (PBS) and fixed with acetone at -20°C for 10 min. Coverslips were washed with PBS, and incubated for expression of von Willebrand factor (vWF) with anti-vWF antiserum (1:50 dilution) (Sigma) and endothelial leukocyte cell adhesion molecule-1 (ELAM-1) (Sigma Life Science) at room temperature for 45 min, and then with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated antibody (Pharmigen, San Diego, CA) for 45 min at 4°C. The cells were washed twice with PBS and 2% FCS and analyzed on a Beckton & Dickinson FACSscan.

Endothelial Cell Proliferation Measurement

Endothelial cell proliferation was measured by the 3-(4,5-dimethylthiazolyl)-2-5-diphe-ny-^{2}H-tetrazolium-bromide (MTT) assay. Endothelial cells were seeded in a gelatin-coated 96-well plate at a cell density of 10^3 cells/well. Cells were grown in RPMI 1640 containing 20% FCS plus 100 μg/ml ECGS (Sigma). After 1, 3, 5, and 7 days of culture, cells were incubated with the MTT solution (0.5%, 50 μl/well) for 4 h. During this incubation period, a water-insoluble formazan dye is formed. After solubilization, the formazan dye is quantitated using a scanning multiwell spectrophotometer (ELISA reader) at 570 nm. The absorbance revealed directly correlates to the cell number. The results were expressed as optical density (OD) at 570 nm. IEC proliferation rate was compared to that of endothelial cells belonging to thoracic aorta (TEC). Semiquantitative Reverse Transcription-Polymerase Chain Reaction for messenger RNA of Api in endothelial cells: Isolated endothelial cells from islets and from thoracic aorta and large liver vessels (used as controls) were seeded in a gelatin-coated six-well plate (5×10^5 cells/well). After overnight culture, total RNA from these cells was extracted using TRIzol reagent (Life Technologies, GIBCO-BRL, Milan, Italy). After treating with RNAse-free DNAseI (Clontech, P.H. Stehelin AG, Basel, Switzerland), reverse transcription (RT) of RNA and polymerase chain reaction (PCR) amplification of cDNA were carried out using the Access RT-PCR kit (Promega/Catalys AG, Wallisellen, Switzerland). Briefly, RT of 0.5 μg of total RNA was performed at 48°C for 45 min, followed by heat inactivation of reverse transcriptase at 94°C for 2 min. The conditions for PCR were the

following: denaturation for 30 s at 94°C, annealing for 30 s at 54°C, and elongation for 30 s at 72°C with 30 cycles. Semiquantitative PCR amplification was carried out on the cDNA from each animal using 3 μl of each dilution of cDNA in a 20 μl reaction with 80 ng of each primer, 0.25 mM of each dNTP, 2.5 μCi of (α-^{32}P) dCTP (3,000 Ci/mmol; DuPont-NEN, Milan, Italy), 1 U of AmpliTaq (Perkin-Elmer/Cetus, Monza, Italy), and 3 mM $MgCl_2$. First-strand cDNA was PCR-amplified with specific oligonucleotide pairs for Api: (forward) 5′-CAATATCTTCTTCTCCCC-3′ and (reverse) 5′-ATGCCTAAACGCTTCATC-3′; and for GAPDH: (forward) 5′-CATGTTCGTCATGGGTGTGA-3′ and (reverse) 5′-AGTGAGCTTCCCGTTCAGCT-3′, used as internal control. PCR products were visualized under ultraviolet light after electrophoresis on 1% agarose gels containing ethidium bromide. The RT-PCR analyses were made in triplicate using a PTC-100 thermal cycler (MJ Research, Watertown, MA). The amplification products were separated on a 1.5% agarose gel containing ethidium bromide, and compared with DNA reference markers. The intensities of the bands were quantified in an Ultrascan XL Enhanced Laser densitometer (LKB, Bromma, Sweden) and expressed in arbitrary units of OD.

Statistical Evaluation

Data were computed as means ± SD and compared using Student's *t*-test for paired or unpaired samples, as appropriate.

RESULTS

Blood Glucose Levels

As shown in Table I, NOD WT mice had normal blood glucose levels up to week 10, then their levels rose by 15 weeks, although animals were not considered hyperglycemic, since glycemia remained below 8 mmol/L. NOD WT mice become overtly diabetic by week 25, when their blood glucose levels considerably exceed 12 mmol/L. NOD IL-10-deficient mice were hyperglycemic by week 15, then by week 20 borderline diabetic and overtly diabetic at week 25. Transgenic NOD-*Il10*^{tm1Cgn} mice only showed a slight hyperglycemia by week 15, and by week 20, they were hyperglycemic; at week 25, they only showed a slight further progression of blood glucose values. Control C57BL/J mice were always normoglycemic.

Insulitis and Grading Score Assessment

As shown in Table II, insulitis grading scores increase with the age of WT NOD mice. In particular, at week 10, animals were infiltrated by a peri-insulitis and a vasculitis, while still euglycemic. At week 20, this insulitis became an intra-islet insulitis (when they were hyperglycemic), and then further progressed towards islet atrophy and cytoarchitectural derangement at week 25, when the animals were overtly diabetic. IL-10-deficient NOD mice showed a similar degree of infiltration. Conversely, transgenic NOD mice showed a much slower progression in the degree of infiltration (see Table II). C57 nondiabetic controls never showed signs of infiltration.

Ex-Vivo Api Expression

Observations made ex vivo on freshly isolated pancreata confirmed that the expression of Api is only observable in islet endothelia of young pre-diabetic WT NOD (Fig. 1a). The expression intensity of Api, as shown by immunofluorescence, decreased with the age of the animals (Fig. 1b). Moreover, it was never observed in IL-10-deficient NODs, although an increased expression was observed in transgenic NOD-*Il10*^{tm1Cgn} mice (Fig. 1c).

TABLE I. Blood Glucose Levels in NOD Wild Type (NOD WT), NOD-*Il10*^{tm1Cgn} (Transgenic), and C57B16/J

	Week 10	Week 15	Week 20	Week 25
NOD WT	5.2 ± 0.2	7.4 ± 0.5[a]	9.2 ± 0.5[b]	18.8 ± 1.0[c]
NOD IL-10$^{-/-}$	5.5 ± 0.4	8.5 ± 1.1[a]	11.2 ± 2.5[c]	24.5 ± 2.5[c]
NOD-*Il10*^{tm1Cgn}	5.6 ± 0.5	7.0 ± 0.5[a]	8.0 ± 1.2[b]	9.1 ± 1.8[b]
C57	—	—	—	—

Values, expressed as mmol/L, are given as means ± SD.
[a]$P < 0.01$ vs. week 10.
[b]$P < 0.001$ vs. week 10.
[c]$P < 0.0001$ vs. week 10.

TABLE II. Insulitis Grading Score in NOD Wild Type (NOD WT), NOD-*Il10*^{tm1Cgn} (Transgenic), and C57B16/J

	Week 10	Week 15	Week 20	Week 25
NOD WT	1.2 ± 0.2	3.0 ± 0.3[a]	4.0 ± 0.4[a]	4.6 ± 0.2[a]
NOD IL-10$^{-/-}$	1.4 ± 0.2	3.5 ± 0.5[a]	4.4 ± 0.6[a]	5.0 ± 0.1[a]
NOD-*Il10*^{tm1Cgn}	0.8 ± 0.2	1.2 ± 0.2	1.9 ± 0.3	2.0 ± 0.4[b]
C57	—	—	—	—

Data are given as means ± SD.
[a]$P < 0.0001$ vs. week 10.
[b]$P < 0.001$ vs. week 10.

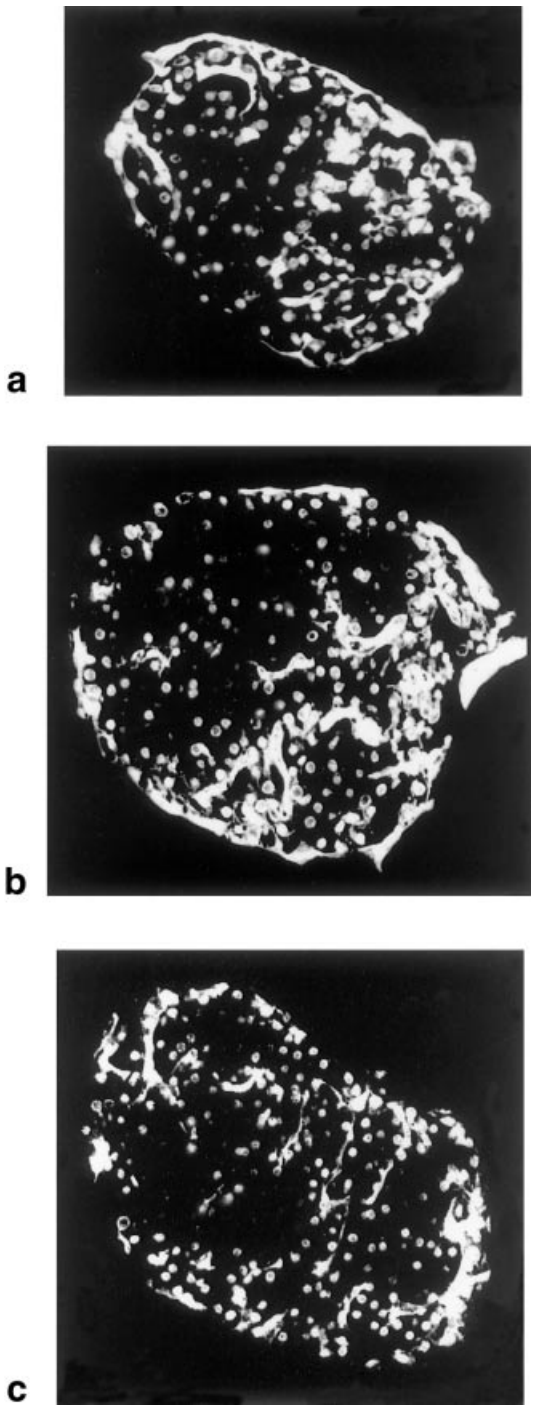

Fig. 1. Light micrograph representative of Api expression in freshly isolated pancreatic tissues. **a**: Anti-Api immunoreactivity of islet endothelia in a pancreatic islet belonging to a 10-week-old NOD animal (original magnification 350 ×); (**b**) anti-Api immunoreactivity of islet endothelia in a pancreatic islet belonging to a 20-week-old NOD animal (original magnification 350 ×); (**c**) anti-Api immunoreactivity of islet endothelia in a pancreatic islet belonging to a 15-week-old transgenic NOD-*Il10*^{tm1Cg}mice (original magnification 350 ×).

Characterization of IEC

Immunocytochemistry confirmed the high purity of the IEC cultures in which cells obtained from WT, IL-10-deficient NODs, and transgenic NOD-*Il10*^{tm1Cgn} mice expressed both vWF and ELAM-1 molecules. The FITC fluorescence profiles are shown in Figure 2, where more than 90% of cultured cells express positivity to vWF and endothelial leukocyte adhesion molecule-1. No age variation was found during the experiments. The positivity was highly specific as the remaining population of non-positive 10% cells were also negative for Api expression.

Endothelial Cell Proliferation Capacity

Proliferation rate in cultured endothelial cells was quantified by BrdU specific ELISA method. Significant cell proliferation was observed around adherent islets as well as from purified IEC. MTT assay reveals that IEC show a proliferation capacity which was slightly higher ($P < 0.01$), but comparable to that of the thoracic type (TEC). Results obtained from IL-10-deficient or transgenic NOD mice were comparable.

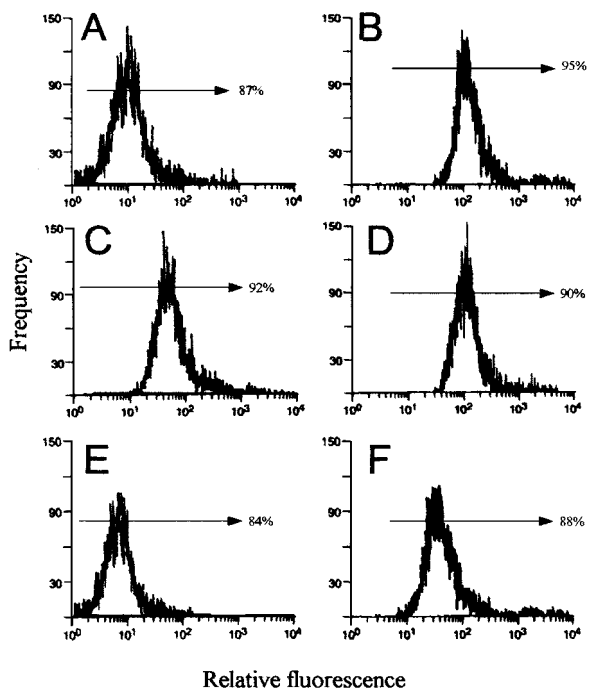

Fig. 2. Representative flow cytometric analysis of ELAM-1 (**A**, **E**) and vWF (**B**, **C**, **D**, **F**) endothelia from wild type NOD (A, B), IL-10-deficient NODs (**c**), transgenic NOD-*Il10*^{tm1Cg} mice (**d**), and C57Bl6/J controls (E, F) islets showing high positivity (roughly 90% in all the experiments). The figure is representative of three independent experiments.

Expression of Api by Semiquantitative RT-PCR

IEC from 10-week-old prediabetic WT NOD express Api, since a significant band is observed in islet endothelia cultured from these animals (Fig. 3). This band is not found in IEC from nondiabetic control C57BL/J animals. The specificity for IECs is confirmed by the absence of the band in endothelia cultured from thoracic aorta (TEC) and liver (LIEC) (Fig. 3). Interestingly, Api expression is modulated by hyperglycemia in the WT NOD mice. Actually, IEC belonging to 15-week-old WT animals showed a reduction in the intensity of the Api band when compared to that of IEC from 10-week-old mice, and was further reduced by 20 weeks of age (see Table III, showing optical densities) ($P < 0.0001$ vs. 10-week-old NOD WT IEC animals). The band disappeared at 25 weeks, demonstrating that IEC of diabetic animals do not express the specific RNA messenger. Moreover, this band was not observed in IEC from 10-week-old WT NOD cultured in the presence of IL-1β (a Th1 cytokine exerting diabetogenic and specific cytotoxic effects upon islet β cells). Oppositely, IEC obtained from diabetic WT NOD and cultured in the presence of IL-10 (a Th2 protective cytokine, which also exerts vascular effects) clearly show a band of Api mRNA ($P < 0.001$ vs. all groups except NOD IEC week 10, see Table III). IEC cultured from NOD IL-10-deficient mice never showed the specific Api band both under control and experimental conditions, except when their islets were cultured with the addition of IL-10 to the medium. Oppositely, cells from transgenic NOD-*Il10*^{tm1Cgn} mice overexpressed Api at all the time-points and did not show this band only upon challenge with IL-1β. All the above reported results were confirmed when the experiments were repeated (in triplicate).

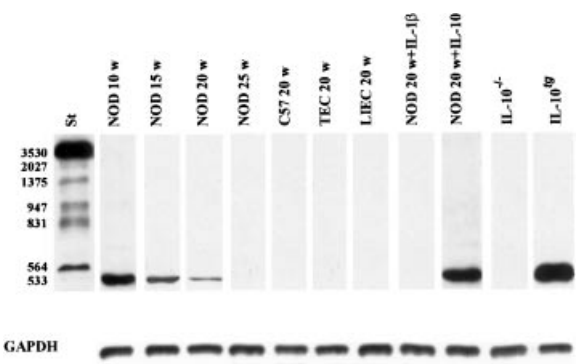

Fig. 3. Figure showing representative RT-PCR for mRNA of Api on IEC from WT, IL-10-deficient (IL-10$^{-/-}$) NODs, transgenic NOD-*Il10*^{tm1Cgn}, and on endothelial cells from TEC and LIEC. **Lane 1**: Cultured IEC from 10-week-old NOD WT; **lane 2**, cultured IEC from 15-week-old NOD WT; **lane 3**, cultured IED from 20-week-old NOD WT; **lane 4**, cultured IEC from 25-week-old NOD WT; **lane 5**, cultured IEC from 20-week-old C57BL6/J; **lane 6**, cultured TEC from 20-week-old NOD WT; **lane 7**, cultured LIEC from 20-week-old NOD WT; **lane 8**, cultured IEC from 20-week-old NOD WT + IL-1β; **lane 9**, cultured IEC from 20-week-old NOD WT + IL-10; **lane 10**, cultured IEC from 20-week-old NOD- IL-10 $^{-/-}$; **lane 11**, cultured IEC from 20-week-old NOD-*Il10*^{tm1Cgn} (transgenic).

TABLE III. Optical Densities, Expressed in Arbitrary Units ± SD, of the Api PCR Bands (Means of Three Independent Experiments)

	10 W	15 W	20 W	25 W
NOD IEC	5.9 ± 0.4^a	2.8 ± 0.3	2 ± 0.3	0.7 ± 0.2
NOD TEC	0	0	0	0
NOD LIEC	0	0	0	0
NOD + IL-1β	0	0	0	0
NOD + IL-10	6.5 ± 0.9^b	5.6 ± 0.5^b	5.1 ± 0.5^b	4.4 ± 0.3^b
NOD IL-10$^{-/-}$	0	0	0	0
NOD + IL-10^{tg}	6.6 ± 0.8^c	6.5 ± 0.6^c	6 ± 0.6^c	6.2 ± 0.4^c
C57	0	0	0	0
GAPDH	5 ± 0.2	4.9 ± 0.2	4.7 ± 0.2	4.9 ± 0.3

[a] $P < 0.001$ vs. weeks 15, 20, 25.
[b] $P < 0.001$ vs. all groups except NOD IEC week 10.
[c] $P < 0.001$ vs. NOD IL-10$^{-/-}$ and NOD WT 20 weeks.

DISCUSSION

This study clearly demonstrates that, like in humans [Lou et al., 1999], the Api is highly specific for islet microvascular endothelial cells obtained from NOD mice, since it is absent in endothelia of other organs. Interestingly, the present work also shows that Api expression is strongly modulated by cytokines, leading to the thought that this proteinase inhibitor may be also involved in the progression of the anti-islet β cell attack. Actually, the mRNA band, clearly observable in prediabetic, still euglycemic (but infiltrated) WT NOD mice, is slightly reduced in 15-week-old NOD animals, which also show a significant rise in glycemia; thereafter, in clearly hyperglycemic 20-week-old animals, its intensity is reduced dramatically and disappears all together in diabetic NOD mice of 25 weeks of age. That it may have a specific role during the early anti-islet β cell attack is further suggested by the other findings shown in this study. In fact, IEC cultures obtained from animals at all time points and challenged with IL-1β, (a Th1 proinflammatory cytokine, which has been found to be a critical effector molecule

in type 1 diabetes occurring in the NOD mouse [Cailleau et al., 1997]), never show the Api band, suggesting that IL-1β downregulates its activity. On the contrary, addition of IL-10 (a Th2 cytokine) to IEC cultures, increases the expression of Api, which remains clearly observable also in IEC from 20-week-old NOD animals, thus demonstrating that IL-10 upregulates Api expression. Moreover, IL-10-deficient NODs do not show the Api band (except after exogenous addition of IL-10), confirming that this cytokine is needed to specifically upregulate Api expression. However, these animals in our experiments showed a slightly accelerated progression of diabetes as assessed by glycemia and insulitis grading score. The paradoxical role of IL-10 is not new, since this cytokine, albeit thought Th2 protective, exerts contrasting effects in the immunoregulation of autoimmune diabetes [Balasa and Sarvetnick, 1996]. Moreover, it has been already found that IL-10 deficiency does not inhibit insulitis and does not significantly modify the course of hyperglycemia and diabetes [Balasa et al., 2000a]. On the other hand, our findings on Api modulatory effects were further substantiated by the use of transgenic NOD-*Il10*^{tm1Cgn} mice, which showed a much slower progression of the disease, as well as an overexpression of Api.

Therefore, in this study, IL-10 exibits a modulatory effect upon Api, upregulating its expression. This property would be partly derived by the endothelial cell activation described for this cytokine [Pennline et al., 1994; Wogensen et al., 1994; Gunnett et al., 2000] and may contribute to the understanding of its debated role in type 1 diabetes [Balasa and Sarvetnick, 1996; Balasa et al., 2000b]. Anyway, it is a point of interest which could be taken into consideration among the main activities of said cytokine in blocking the immune response.

Furthermore, Api is thought to be not only a proteinase inhibitor, but also an immune effector by modulating lymphocyte proliferation, cytotoxicity, transendothelial migration, and monocyte and neutrophyl functions [Breit et al., 1985]. Its expression is highly significant in islet homeostasis because it may be involved in islet neovascularization and revascularization after transplantation, and its appearance may favor or accelerate autoimmune disorders [Bristow et al., 1998; Sandler et al., 1998]. Our results stress this role, mainly on the light of the upregulatory activity exerted by IL-10. In fact, this cytokine, which has been found to be associated with high IL-4 levels in NOD mice [Hancock et al., 1995] and has been implicated as diabetes preventive [Balasa et al., 2000a,b], in addition to anti-inflammatory and immunosuppressive properties [Moore et al., 1993], acts as a potent recruitment signal for leukocyte migration [Pennline et al., 1994], and is also capable of activating endothelia [Pennline et al., 1994; Gunnett et al., 2000]. The latter is an interesting point, which needs further research, also in view of the involvement of islet endothelia and vessels in type 1 diabetes development [Pober and Cotran, 1990; Papaccio and Chieffi Baccari, 1992; Papaccio et al., 1998].

On the other hand, the lack of expression of Api mRNA transcripts in control nondiabetic animals (C57Bl6/J), rather than unexpected, may be interpreted as a further evidence in favor of the hypothesis that this proteinase inhibitor is expressed only during the prediabetic state or when ongoing diabetes is taking place; in fact its expression is always detectable in prediabetic animals, in animals treated with a protective antiinflammatory cytokine, and in those that overexpress this cytokine (transgenic animals) and declines with diabetes appearance. It is absent in IL-10-deficient or in IL-1β treated animals, thus strongly demonstrating its major role during the prediabetic condition and when protection against ongoing diabetes is occurring.

Our data, taken together, suggest that the expression of Api on IEC plays a pivotal role during the early appearance of type 1 diabetes. Moreover, these findings emphasize that, other than diabetes itself, cytokines are involved in the regulation of Api expression.

ACKNOWLEDGMENTS

Authors are grateful to Prof. V. Nigro and his coworkers (TIGEM Laboratory for Genetics, Naples, Italy) for expert technical assistance and to Mr. MGV Latronico (I.N.M. Pozzilli, IS, Italy) for animal backcrossing and help.

REFERENCES

Balasa B, Sarvetnick N. 1996. The paradoxical effects of interleukin 10 in the immunoregulation of autoimmune diabetes. J Autoimmun 9:283–286.

Balasa B, Van Gunst K, Jung N, Katz JD, Sarvetnick N. 2000a. IL-10 deficiency does not inhibit insulitis and accelerates cyclophosphamide-induced diabetes in the nonobese diabetic mouse. Cell Immunol 202:97–102.

Balasa B, Van Gunst K, Jung N, Balakrishna D, Santamaria P, Hanafusa T, Itoh N, Sarvetnick N. 2000b. Islet-specific expression of IL-10 promotes diabetes in nonobese diabetic mice independent of Fas, perforin, TNF receptor-1, and TNF receptor-2 molecules. J Immunol 165:2841–2849.

Bonner Weir S, Orci L. 1982. New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes 31:883–889.

Breit SN, Wakefield D, Robinson JP, Luckhurst E, Clark P, Penny R. 1985. The role of alpha 1-antitrypsin deficiency in the pathogenesis of immune disorders. Clin Immunol Immunopathol 35:363–380.

Bristow CL, Di Meo F, Arnold RR. 1998. Specific activity of alpha 1 proteinase inhibitor and alpha 2 macroglobulin in human serum: application to insulin-dependent diabetes mellitus. Clin Immunol Immunopathol 89:247–259.

Cailleau C, Diu-Hercend A, Ruuth E, Westwood R, Canaud C. 1997. Treatment with neutralizing antibodies specific for IL-1β prevents cyclophosphamide-induced diabetes in nonobese diabetic mice. Diabetes 46:937–940.

Gunnett CA, Heistad DD, Berg DJ, Faraci FM. 2000. IL-10 deficiency increases superoxide and endothelial dysfunction during inflammation. Am J Physiol Heart Circ Physiol 279:H1555–H1562.

Hancock WW, Polanski N, Zhang J, Blogg N, Weiner HL. 1995. Suppression of insulitis in nonobese diabetic (NOD) mice by oral insulin administration is associated with selective expression of interleukin-4 and -10, transforming growth factor-β, and prostaglandin-E. Am J Pathol 147:1193–1199.

Lou J, Triponez F, Oberholzer J, Wang H, Yu D, Buhler L, Cretin N, Mentha G, Wollheim CB, Morel P. 1999. Expression of α-1 proteinase inhibitor in human islet microvascular endothelial cells. Diabetes 48:1773–1778.

Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. 1993. Interleukin-10. Annu Rev Immunol 11:165–190.

Papaccio G. 1993. Insulitis and islet microvasculature in type 1 diabetes. Histol Histopathol 8:751–759.

Papaccio G, Chieffi Baccari G. 1992. Alterations of islet microvasculature in low-dose streptozocin-treated mice. Histochemistry 97:371–374.

Papaccio G, Chieffi Baccari G, Mezzogiorno V, Esposito V. 1990. Capillary area in early low-dose streptozocin-treated mice. Histochemistry 95:19–21.

Papaccio G, Latronico MVG, Pisanti FA, Federlin K, Linn T. 1998. Adhesion molecules and microvascular changes in the nonobese diabetic (NOD) mouse pancreas. An NO-inhibitor (L-NAME) is unable to block adhesion inflammation-induced activation. Autoimmunity 27:65–77.

Pennline KJ, Roque-Gaffney E, Monahan M. 1994. Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. Clin Immunol Immunopathol 71:169–175.

Pober JS, Cotran RS. 1990. The role of endothelial cells in inflammation. Transplantation 50:537–544.

Samols E, Stagner JI, Ewart RB, Marks V. 1988. The order of islet microvascular cellular perfusion is B→A→D in the perfused rat pancreas. J Clin Invest 82:350–353.

Sandler M, Stewart RI, Gemperli BM, Hanekom C, Kuhn SH. 1987. Serum alpha-1 protease inhibitor activity and pulmonary function in young insulin-dependent diabetic subjects. Respiration 52:281–289.

Sandler M, Gemperli BM, Hanekom C, Kuhn SH. 1998. Serum alpha 1-protease inhibitor in diabetes mellitus reduced concentration and impaired activity. Diabetes Res Clin Pract 14:249–255.

Stagner JI, Samols E, Bonner-Weir S. 1988. β→α→δ pancreatic islet cellular perfusion in dogs. Diabetes 37:1715–1821.

Suscheck C, Fehsel K, Krönke K-D, Sommer A, Kolb-Bachofen V. 1994. Primary cultures of rat capillary endothelial cells. Constitutive and cytokine-inducible macrophagelike nitric oxide synthases are expressed and activities regulated by glucose concentration. Am J Pathol 145:685–695.

Wogensen L, Lee MS, Sarvetnick N. 1994. Production of interleukin-10 by islet cells accelerates immune-mediated destruction of β cells in nonobese diabetic mice. J Exp Med 179:1379–1384.