Interleukin-10 up-regulates tumour-necrosis-factor-α-related apoptosis-inducing ligand (TRAIL) gene expression in mammary epithelial cells at the involution stage

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Although interleukin-10 (IL-10) is known to contribute to inflammation and pathogenesis in mammalian organs, little is known about its precise role in the mammary gland. We found that IL-10 levels fluctuated during the mouse mammary cycle, showing little expression at the lactation stage and the highest expression at the involution stage. To reveal the effects of IL-10 on involution, expression profiles of apoptosis-related genes were examined in mice transgenic for IL-10 as well as in IL-10$^{-/-}$ mice. Mild inflammatory lesions by lymphocytes were observed in the mammary glands from four of seven transgenic lines at the lactation stage. It was striking that the expression of tumour-necrosis-factor-α-related apoptosis-inducing ligand (TRAIL) among the apoptosis-related genes was elevated approx. 7-fold in the transgenic mice, whereas others were almost unchanged. Furthermore, TRAIL was down-regulated 4-fold in the IL-10$^{-/-}$ mice at the involution stage. Elevated expression of TRAIL and of death receptor 4 (DR4) protein was identified at the involution stage of normal mammary glands as well as at the lactation stage of the IL-10 transgenic mice. These results indicate that the elevated expression of IL-10 at the involution stage recruits lymphocytes and induces the expression of TRAIL and DR4. These phenomena might partly contribute to apoptosis in the mammary epithelial cells for entering involution.

Key words: apoptosis, death receptor, mammary gland.

INTRODUCTION

Interleukin-10 (IL-10) is a homodimeric protein with a wide spectrum of anti-inflammatory and immune activities [1,2]. It inhibits cytokine production and the expression of immune surface molecules by various cell types [3–5]. This cytokine was also shown to enhance MHC class II antigen expression and to induce the proliferation and differentiation of B cells [6]. In addition, IL-10 induces the expression of cell adhesion molecules on endothelial cells and the apoptotic cell death of B-chronic lymphocytic leukaemia cells [7]. These properties of IL-10 indicate that it is a potent immunosuppressant and has great potential therapeutic utility in the treatment of diseases, such as chronic inflammation, autoimmune diseases, transplant rejection, graft-versus-host disease and sepsis [8–10]. Mice carrying a null mutation in both alleles of the gene encoding IL-10 showed normal lymphocyte development and antibody responses but also growth retardation and chronic enterocolitis, demonstrating an essential role for the protein in immunoregulation in the intestinal tract [11].

In the salivary and lacrimal glands, it has recently been found that IL-10 induced apoptosis of the cells via the Fas pathway [12]. Expression of IL-10 induced the expression of Fas-L in CD4$^+$ T-cells, which interact with and kill the Fas-expressing cells. The results suggested that overexpression of IL-10 in the glands might be a causal factor in the development of Sjogren's syndrome [13].

Being different from other exocrine gland cells, mammary cells cycle through growth, differentiation and involution during mammals' fertile age. During the growth and differentiation of epithelial cells, a combination of hormones and growth factors including oestradiol and progesterone is important [14,15]. Involution is characterized both by the apoptosis of mammary epithelial cells and by tissue remodelling. During this period, marked changes in gene expression occur after the cessation of milk removal; massive apoptosis begins. Several death-related genes are activated at this stage, and a process of cell shrinkage, DNA fragmentation and nuclear condensation occurs [16–18]. However, compared with growth and differentiation, the pathway by which apoptotic mammary epithelial cells are cleared from the gland is not well understood.

In human milk, various cytokines including tumour necrosis factor α (TNF-α), transforming growth factor β1 (TGF-β1), IL-1β and IL-6 were expressed at a physiologically significant level [19]. They act as immunoregulators or as developmental modulators. For example, TGF-β1 regulates the development and function of the alveolar structures [20]. Transgenic females that express TGF-β1 in the mammary glands were unable to lactate,

owing to inhibition of the formation of lobuloalveolar structures and suppression of the production of endogenous milk protein. Expression of IL-6 is significantly decreased in invasive ductal carcinoma in comparison with normal mammary tissue and with lesions *in situ*. Conversely, the expression of IL-6 in invasive lobular carcinoma was enhanced [21]. IL-10 has been also found in human milk at concentrations from 60 to 9300 pg/ml, suggesting that the levels were physiologically significant [19]. However, its precise function in the mammary gland remains elusive. In the present study, to elucidate the function of IL-10 in the mammary gland, we examined its expression profile through the stages of the mammary cycle in mice. Furthermore, the effect of IL-10 on the expression of apoptosis-related genes was investigated in transgenic mice by expressing human IL-10 (hIL-10) in mammary epithelial cells at lactation and also in IL-10$^{-/-}$ mice.

MATERIALS AND METHODS

Mice

ICR and BCF1 mice were purchased from the Genetic Resource Center at Kribb (Taejon, Korea), whose animal facility is authorized by the Institute of Laboratory Animal Resources (National Research Council, Washington, DC, U.S.A.). C57BL/6 IL-10$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). In all experiments *in vivo*, groups consisted of three or more animals.

Semi-quantitative reverse-transcriptase-mediated PCR (RT–PCR)

ICR mice were mated naturally, and the same-site tissues among the eight mammary glands were collected at days 6.5 and 12.5 of pregnancy, at days 2 and 5 of lactation and at days 2, 5 and 8 of involution. RNA was prepared from at least three mice per mammary stage. Reverse transcription was performed for the total RNA of mammary glands or HC11 cells with a reverse transcription kit (Promega). The expression level of mouse IL-10 (mIL-10) was measured by PCR with primers specific for *mIL-10* cDNA. The primers were 5′-GCTGAGGCGCTGTCATCGAT-3′ and 5′-GAGCTGCTGCAGGAATGATC-3′, amplifying a 679 bp fragment of *mIL-10* cDNA. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers were used for the normalization of IL-10 expression. The G3PDH primers were 5′-ACCA-CAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTG-CTGTA-3′, amplifying the 500 bp cDNA fragment. [^{32}P]dCTP (Amersham Pharmacia Biotech) was added to the reaction and the PCR products were detected by autoradiography. PCR reactions were performed for 25 cycles at 94 °C for 45 s, 62 °C for 1 min and 72 °C for 40 s.

Construction of an IL-10 expression vector

The full-length human gene encoding IL-10 was cloned by screening a human genomic library constructed in the EMBL3 vector (ClonTech). The probe DNA was prepared by PCR amplification of the 384 bp DNA fragment spanning the proximal promoter and exon 1 of the gene encoding hIL-10 (GenBank® accession no. U16720) using the chromosomal DNA of blood samples. The primers for the PCR were 5′-TGCAGAAGTTC-ATGTTCA-3′ and 5′-TCTCGAAGCATGTTAGGC-3′. The positive phage DNA insert was subcloned into the *Sal*I site of the pBluescript II SK vector.

An IL-10 expression cassette was constructed by combining the bovine β-casein promoter and the gene encoding hIL-10. In brief, 12 kb of the IL-10-containing *Sph*I–*Sal*I DNA fragment

was subcloned into a pBluescript II SK vector containing 10 kb of the bovine β-casein promoter [22]. An adapter was linked into the *Sph*I site to insert a *Sac*II site. The nucleotide sequence of the adapter was 5′-CCTCCGCGGAGGCATG-3′.

Generation of transgenic mice

The DNA for microinjection was prepared by digesting the IL-10-expressing plasmid vector with *Sac*I and *Sal*I; the larger fragment was purified by passing it through a 0.8 % agarose gel and an Elutip column (Schleicher and Schuell). Transgenic mice were generated with a standard method described previously [23], with minor modifications. The mice were an F$_1$ hybrid C57BL/6 × CBA strain. Founder mice and/or their offspring were analysed for inheritance, copy number and expression of the transgene.

Northern blot analysis

Total RNA from various organs (mammary gland, brain, heart, lung, kidney, liver, spleen and pancreas) was purified with Trizol RNA purification solution (Gibco BRL). For Northern blot analysis, 30 μg of total RNA was separated on 1.0 % (w/v) agarose/formaldehyde gels and transferred to nylon membranes (Roche Molecular Biochemicals). The blots were probed with a 164 bp exon 1 of the IL-10 cDNA that was amplified by RT–PCR with primers 5′-ATGCACAGCTCAGCACT-3′ and 5′-AAAG-AAAGTCTTCACTC-3′. The probe was labelled by the random-priming method with a DNA labelling kit (Amersham). Hybridizations were performed for 4 h in Quick Hybridization solution (ClonTech) at 65 °C. The filters were then washed twice at 65 °C in 0.1 × SSC/0.1 % SDS for 20 min and exposed to X-ray films for 24–48 h at −70 °C.

Ribonuclease protection assay (RPA)

Total RNA species were extracted from mammary glands of mice with Trizol reagent. Apoptosis-related mRNA species were detected with a RiboQuant Multiprobe RPA system (PharMingen). In brief, riboprobes were labelled with ^{32}P and hybridized overnight with 20 μg of the RNA samples. The hybridized RNA was treated with RNase and purified in accordance with the RiboQuant protocol. The protected fragments were resolved by electrophoresis on a 5 % (w/v) polyacrylamide/urea gel; autoradiograms were exposed for 48 h.

Analysis of protein in milk and tissues

Milk was collected at day 10 of lactation as reported previously [24]. For the Western blot analysis, whey was diluted 1:3 to 1:12 and 1 μl was mixed with electrophoresis sample buffer [2 % (w/v) SDS/10 % (v/v) glycerol/0.08 M Tris/HCl (pH 6.8)/2 mM EDTA/0.1 M dithiothreitol/0.01 % Bromophenol Blue] and denatured at 95 °C for 10 min before loading on a 15 % (w/v) denaturing polyacrylamide gel. For the preparation of proteins from mammary tissues, the tissues were homogenized with PBS and centrifuged at 4 °C for 10 min at 12000 *g*. The supernatant was used for the immunoblot analysis. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Polyclonal goat anti-(hIL-10) antiserum (diluted 1:1000 in 1 % bovine serum albumin) (Santa Cruz Biotechnology), polyclonal rabbit antibody against TNF-α-related apoptosis-inducing ligand (TRAIL), and monoclonal mouse antibody against death receptor 4 (DR4) (1:5000 dilution) (Santa Cruz Biotechnology Inc.) were used to detect hIL-10, TRAIL and DR4 respectively

by Western blot analysis. Bound antibody was detected by addition of alkaline phosphatase-conjugated goat anti-(goat IgG) (Bio-Rad) for IL-10 or horseradish-peroxidase-conjugated antibody (Amersham Life Science) for TRAIL and DR4. For the normalization of protein expression, mouse β-actin or lactoferrin was examined by using its monoclonal antibody (Sigma). For ELISA analysis, milk samples were diluted $1:10^5$ to $1:10^6$ and the IL-10 expression levels were determined with an hIL-10 ELISA (PharMingen) and a mIL-10 ELISA kit (R & D Systems) in accordance with the supplier's protocol.

Measurement of TNF-α in macrophage culture

Peritoneal macrophages from Balb/c mice were isolated by flushing the peritoneal cavity with 7 ml of RPMI medium with a 22-gauge syringe. Approximately 2×10^5 cells were plated on a well of the 96-well culture dish and incubated at 37 °C for 6 h. The cultured cells were then stimulated with 2 μg/ml endotoxin (derived from 055:B5 *Escherichia coli*; Sigma) in the presence of mouse milk or recombinant hIL-10 (Sigma). Supernatants were collected at 18 h after challenge with endotoxin and were examined for TNF-α content with an ELISA kit (Endogen).

Histological analysis

Mouse tissues were taken at day 10 of lactation by necropsy and fixed for 24 h in 10 % (v/v) formalin; 4 μm paraffin sections were deparaffinized and hydrated. Sections were stained with haematoxylin and eosin with the standard method [25]. Histological grading of the inflammatory lesions in the mammary gland was done as described by White and Casarett [26].

RESULTS

IL-10 expression is suppressed at lactation and induced at the involution stage during the mammary cycle in mice

As a first step towards understanding the potential roles of IL-10 during the mammary cycle in normal mice, its expression level during the mammary cycle was monitored by the RT–PCR analysis in at least three mice for each developmental stage of the mammary gland. The expression of mIL-10 in the mammary glands was detected at early pregnancy. Expression continued until at least pregnancy day 6.5 (Figures 1A and 1B) and then decreased by pregnancy day 12.5. During lactation days 2 and 5, almost no expression was detected. As the mammary cells were induced to enter the involution stage by separating the pups from their mother, they began to express IL-10 again, and the level reached a peak at day 5 of involution. The expression of IL-10 protein at involution was confirmed by ELISA analysis. The results indicated that its level ranged from 140 to 750 pg/mg of protein at day 5 of involution in the mammary tissue, but remained at basal levels at day 5 of lactation (Figure 1C). These results indicate that the IL-10 expression level fluctuates cyclically during the mammary cycle.

Ectopic expression of bioactive hIL-10 in the epithelial cells of mammary glands during lactation

To explore the possible function of IL-10 in the mammary gland, IL-10 was expressed ectopically during lactation by developing transgenic mice. For this purpose we used bovine β-casein promoter, because it had been shown to be expressed specifically at the lactation stage in transgenic mice (Figure 2A). Eight transgenic mice were obtained from 34 mice analysed by PCR. Total RNA species of various organs from transgenic mice were
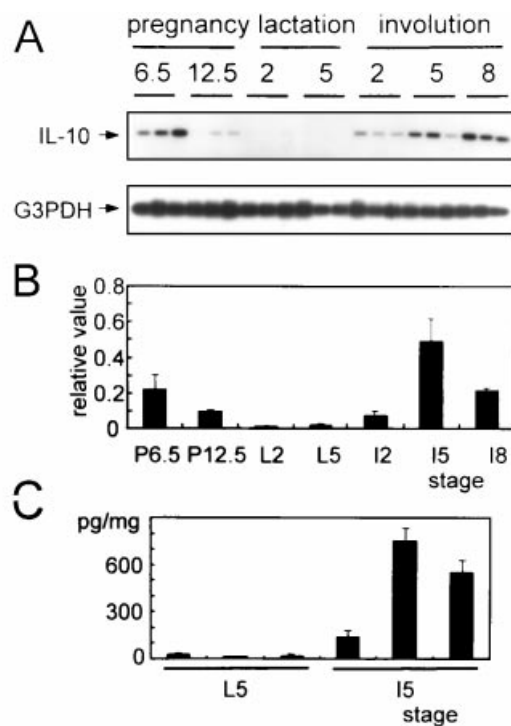

Figure 1   Expression profiles of mIL-10 RNA in the mammary glands during mammary cycles in normal mice

(**A**) The level of mIL-10 was evaluated by semi-quantitative RT–PCR in the pregnancy, lactation and involution stages of the mammary cycle. The numbers below the stage denote the specific day of each stage. Three independent results of three mice per each stage are shown. Mouse G3PDH was used as an internal control. (**B**) Graphical representation of the RT–PCR signals. Quantification was performed with a PhosphoImager. To determine the relative degrees of expression, signal levels were normalized to those of G3PDH; means and S.E.M. are shown. (**C**) Graphical representation of the IL-10 expression level in the mammary gland. The IL-10 level was determined by ELISA at day 5 of lactation (L5) and at involution (I5). For each stage, results for three mice are shown; the expression levels were normalized to protein concentration.

analysed for Northern hybridization. Interestingly, in all the transgenic mice hIL-10 was expressed at detectable amounts, even though the expression level was variable between them (Figure 2B). Results for three transgenic mice are shown in Figure 2(B). The transgene was expressed only in the mammary gland in all transgenic mice except one, which showed the highest expression level. In this mouse the transgene was also expressed in the salivary gland at a much lower level than in the mammary gland (results not shown). In general we concluded that the transgene was expressed specifically in mammary gland, driven by 10 kb of the bovine β-casein promoter.

Protein expression of recombinant hIL-10 in the epithelial cells of the mammary gland was evaluated by immunoblot analysis after milk had been collected on day 10 of lactation. A polyclonal antibody raised against purified IL-10 recognized a polypeptide that appeared only in the transgenic milk; the polypeptide had a molecular mass of approx. 20 kDa, like that of purified hIL-10 (Figure 2C). The expression level of recombinant hIL-10 in milk was estimated by ELISA analysis and ranged from 1.4 to 408 μg/ml.

To determine whether the recombinant hIL-10 has biological activity, its inhibitory effect on TNF-α expression was examined. Macrophages were isolated from the peritoneal cavity of the mouse and plated on culture dishes, then challenged with

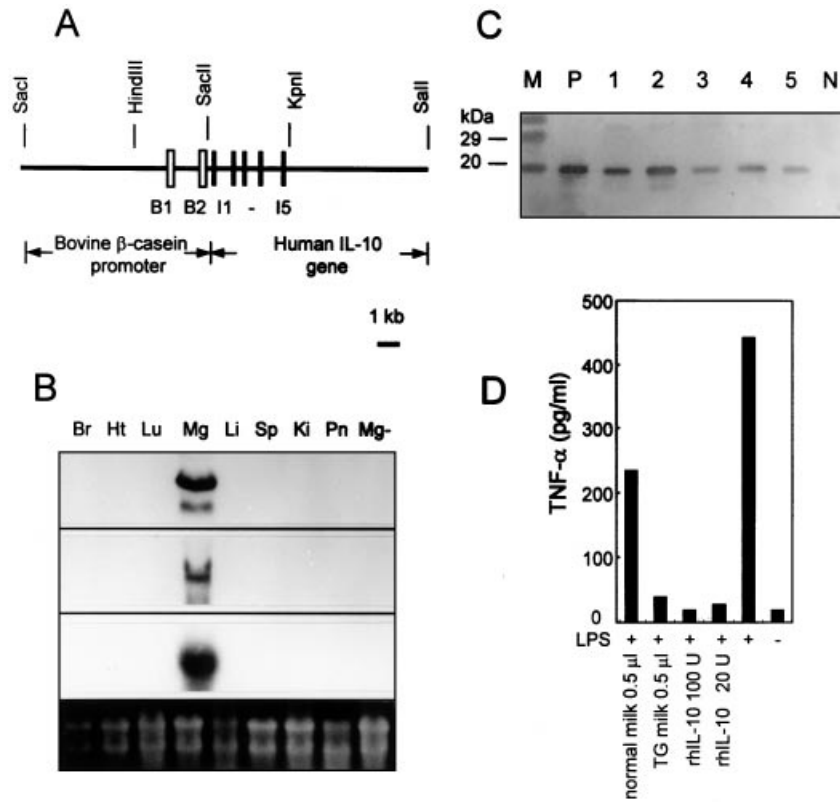**Figure 2   Induction of hIL-10 expression at the lactation stage in the transgenic (TG) mice**

(**A**) An hIL-10 expression vector. The promoter contains 8 kb of the 5′-flanking sequence, the untranslated exons 1 and 2 (vertical open boxes indicated as B1 and B2) and 2 kb of intron 1 of the bovine β-casein gene. The gene encoding hIL-10 contains all of the coding regions spanning from exon 1 to exon 5 (vertical thick lines indicated as I1 to I5) and 7 kb of the 3′-flanking sequences. (**B**) Northern blot analysis of total RNA from tissues of transgenic mice. Tissues analysed were brain (Br), kidney (Ki), liver (Li), mammary gland (Mg), heart (Ht), spleen (Sp), lung (Lu), pancreas (Pa) and mammary gland of a normal mouse (Mg —). Transgenic mouse lines are nos 6, 9 and 20 from the top. Bottom panel: staining of the gel with ethidium bromide, to indicate loading. (**C**) Immunoblot analysis of hIL-10 in milk of transgenic mice. Lane P, 200 ng of purified hIL-10; lanes 1–5, whey from five transgenic mice (nos 6, 8, 12, 19 and 20); lane N, normal whey; lane M, molecular mass markers, whose sizes are indicated at the left. (**D**) Bioactivity of the recombinant hIL-10 expressed in milk of transgenic mice. Mouse peritoneal macrophages were treated with recombinant hIL-10 (20–100 i.u./ml) or milk from transgenic mice and then stimulated with endotoxin (LRS). The resultant supernatants were assayed for murine TNF-α by ELISA. Results are means for three experiments per treatment.

endotoxin in the presence of the recombinant hIL-10. Supernatants were collected at 18 h after endotoxin challenge and were examined by ELISA for TNF-α content. The result indicated that the protein properly decreased the TNF-α level in a dose-dependent manner, similarly to the commercial one (Figure 2D).

These results imply that a hybrid transgene comprising 10 kb of the bovine β-casein gene promoter and 12 kb of the gene encoding hIL-10 induced proper levels of the recombinant protein in the milk of transgenic mice in a mammary-specific pattern.

IL-10 induces mild infiltration of lymphocyte into the mammary gland

Tissue sections from 8-week-old transgenic female mice at day 10 of lactation were prepared and stained with haematoxylin/eosin. Histological findings indicated that four of seven transgenic mice had minor inflammatory infiltration in the mammary glands. Two lines also had inflammatory infiltration in the salivary glands; RT–PCR revealed that these mice were expressing the transgene slightly in the salivary gland (results not shown). Figures 3(B) and 3(C) show the histological findings in mammary glands with inflammatory infiltration from two different transgenic lines. No significant infiltration of mono-

nuclear cells or any histological change was seen in normal (Figures 3A and 3D) and other transgenic tissues, except for the salivary glands (Figures 3E and 3F).

IL-10 induces TRAIL in the mammary gland

To address the role of IL-10 during the mammary cycles of the mammary gland, the effects of IL-10 expression on the involution of the glands were examined by monitoring the apoptosis-related gene expression in transgenic mice overexpressing IL-10 as well as in IL-10$^{-/-}$ mice.

In the transgenic mice, the recombinant hIL-10 was properly expressed in the mammary epithelial cells during the lactation stage. When RPA was performed with the mAPO-3 probe kit, expression of the apoptosis-related genes such as caspase, Fas and FasL was increased at the involution stage in both normal and transgenic mice (results not shown). However, we found no great difference in the expression level of these genes between the two mice at the same stage of the mammary cycle, except for TRAIL and TNF-receptor-1 associated death domain (TRADD) protein (Figure 4). In particular, expression of the gene encoding TRAIL, which was identified as a TNF-related apoptosis-
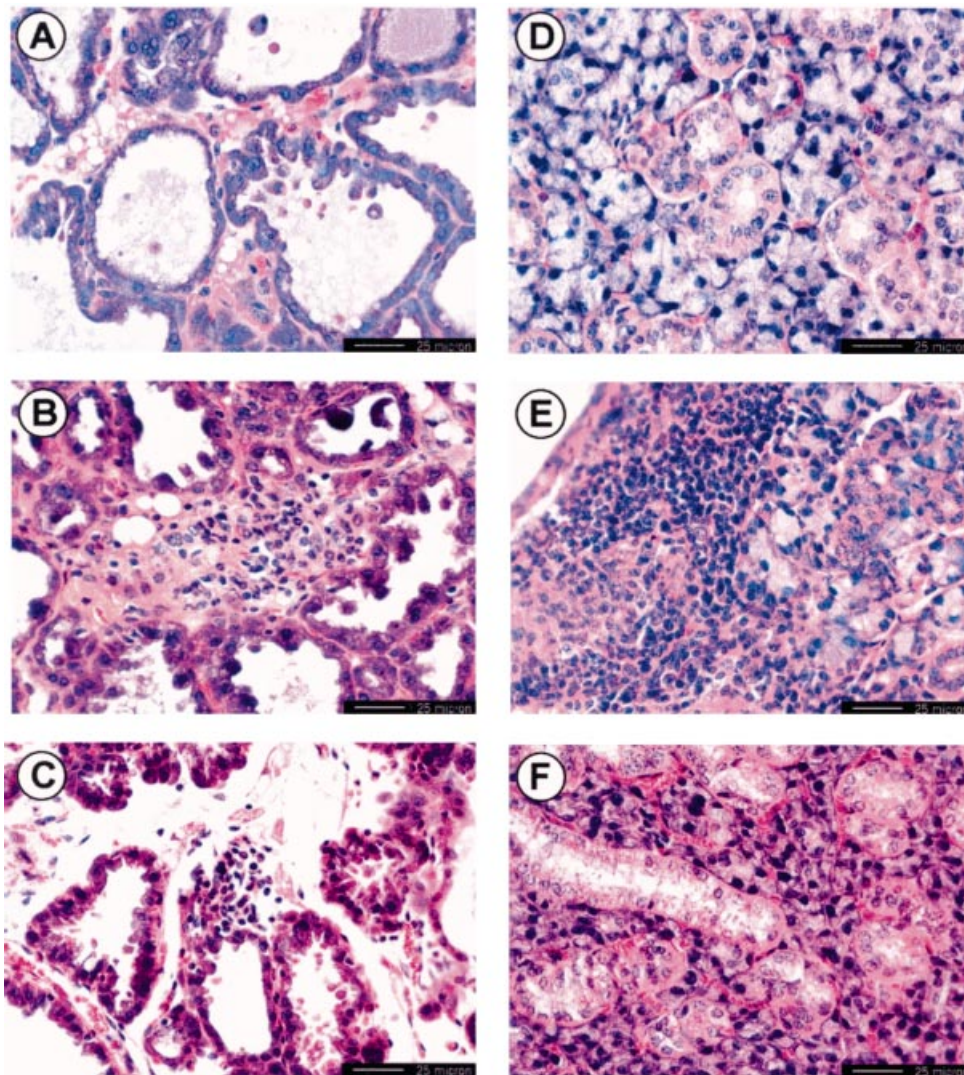
Figure 3   Histological analysis of the sections of the mammary tissues

Sections were prepared from mammary tissues (**A–C**) and salivary glands (**D–F**) of mice on day 10 of lactation, then stained with haematoxylin and eosin. Mild inflammatory lesions were characterized by mononuclear cell infiltration. Four (nos 6, 8, 19 and 200) and two (nos 6 and 19) out of seven mice showed mild chronic inflammatory lesions in the mammary and salivary glands respectively; two transgenic cases were presented. (**A**, **D**) normal tissues; (**B**, **E**) transgenic tissues from mouse no. 6; (**C**, **F**) transgenic tissues from mouse no. 19. Original magnification × 400; scale bar, 25 μm.

inducing ligand [27,28] was increased approx. 4-fold and 7-fold at days 5 and 15 of lactation respectively in the transgenic mice, although its expression was much lower at the lactation stage than at the involution stage, in both normal and transgenic mice. When the mAPO-2 probe kit that included the Bcl-2 family, such as Bax, Bcl-2 and BAD, was used, no specific change in expression for the genes was observed (results not shown).

To characterize the relationship between IL-10 and TRAIL further, the expression of TRAIL was also monitored in C57BL/6 IL-10$^{-/-}$ mice at day 5 of involution. RPA analysis indicated that TRAIL was down-regulated 4-fold in comparison with normal C57BL/6 mice at the involution stage (Figure 5A). These results support the idea that IL-10 expression affects TRAIL expression. In addition to TRAIL, the level of TRADD from mAPO-3 was markedly decreased (Figure 5A). When the Bcl-2 family genes

were examined in these mice by using mAPO-2, Bcl-X$_s$ and Bcl-2 showed marked down-regulation (Figure 5B).

We found that TRAIL protein was induced at the involution stage of the mammary gland and that its expression was affected by IL-10 (Figure 6A). TRAIL was induced in IL-10 transgenic mice but down-regulated in the IL-10$^{-/-}$ mice (Figure 6A, lanes 3 and 4). To examine the effect of TRAIL on the apoptosis of mammary cells, the expression of DR4 protein, which is the receptor of TRAIL and mediates cellular apoptosis, was assayed by Western blot analysis. DR4 was induced as the mammary cells entered the involution stage from lactation (Figure 6B, lanes 1 and 2). The induction of DR4 by IL-10 via TRAIL could be explained by the result that the expression level of DR4 was much higher in the IL-10 transgenic mice than in normal mice (Figure 6B, lanes 3 and 4).
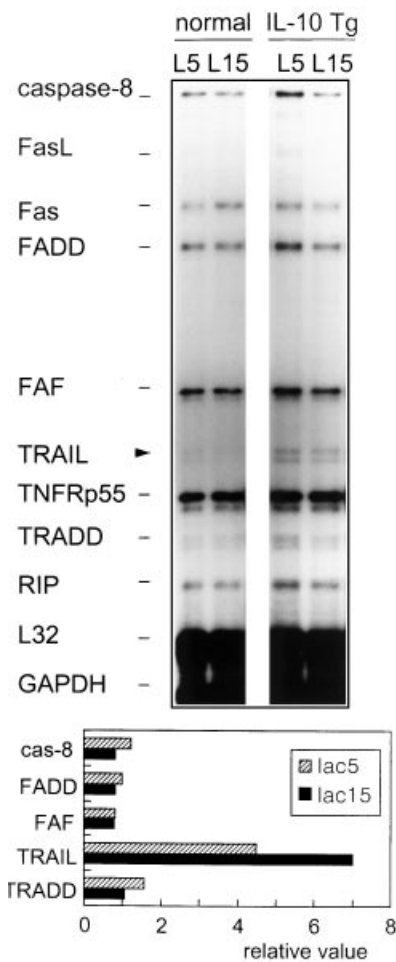
Figure 4   RPA analysis of the mRNA for the apoptosis-related genes in transgenic mice for IL-10

Upper panel: total RNA was isolated from mammary glands of normal and transgenic mice of strain BCF1 at days 5 (L5) and 15 (L15) of lactation, hybridized with ^{32}P-labelled riboprobe with the mAPO-3 probe kit (PharMingen) and developed by autoradiography for 24 h. The expression levels were scanned with a PhosphoImager and compared with control probes (GAPDH and L32). Lower panel: a comparison of the expression levels of a few representative genes, normalized to the L32 housekeeping gene. Levels of TRAIL and TRADD RNA increased approx. 7-fold and 1.6-fold respectively at the lactation stage in the IL-10 transgenic mice.

Figure 5   RPA analysis of the mRNA for apoptosis-related genes in the IL-10$^{-/-}$ mice

Total RNA from mammary glands of normal and IL-10$^{-/-}$ mice of strain C57BL/6 at day 5 of involution was hybridized with ^{32}P-labelled riboprobe with the mAPO-3 kit (**A**) and the mAPO-2 kit (**B**) (PharMingen). Bottom panel: a comparison of the expression levels for a few representative genes, normalized to the L32 housekeeping gene. Level of TRAIL and TRADD (**A**), and Bcl-X$_S$ and Bcl-2 (**B**) were markedly lower in the IL-10$^{-/-}$ mice than in normal mice for the mAPO-3 and mAPO-2 genes respectively.

DISCUSSION

Although IL-10 has been found in human milk [19], its precise effect on mammary cells has not been characterized yet. As a first step towards explaining the function of IL-10 in the mammary gland, we have chased its expression during the normal mammary cycle of the mouse and in mammary cell culture *in vitro*. Next, we examined the effect of IL-10 in transgenic mice overexpressing IL-10 as well as in IL-10-null mutant mice by studying changes in histology and in apoptosis-related genes.

It is known that in secretory glands such as the salivary and lacrimal glands, IL-10 induces lymphocyte infiltration and subsequent Fas/FasL-mediated tissue destruction [12]. However, in the mammary gland, which cycles through cell growth, differentiation and involution, the origin of the IL-10 and its role have not been addressed yet, even though human milk contains much more IL-10 (60–9300 pg/ml) than the organ-cultured salivary or lacrimal glands of mice (less than 40 pg/ml). So far, no disease
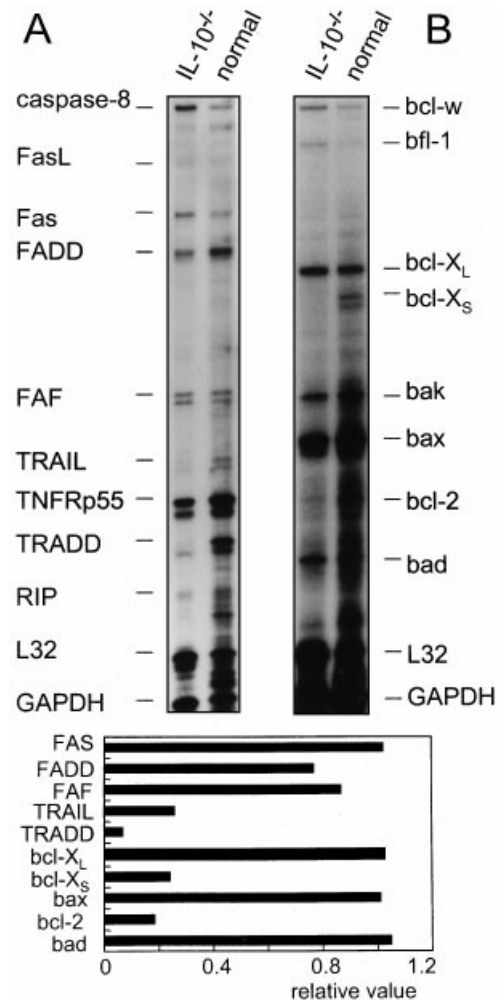
or destruction of mammary tissue caused by IL-10 expression has been reported, and we speculated that IL-10 in the mammary gland could confer its effect by participating in the normal mammary cycle rather than by invoking disease.

IL-10 found in the mammary glands might originate from both the lymphocytes and the epithelial cells. We could not detect IL-10 receptors [29] by RT–PCR analysis on a lymphocyte-free cell culture (HC11) that was originated and established from mouse mammary epithelial cells at mid-pregnancy (results not shown). It is therefore possible that IL-10 synthesized by the epithelial cells cannot act directly on them. Instead, it might induce the infiltration of lymphocytes, as shown in Figure 3, by inducing cell-adhesion molecules on blood vessels [30,31] and acting on their cell-surface receptors. This is reminiscent of the phenomenon in transgenic mice in which IL-10 is targeted to the salivary gland. However, the occurrence of inflammatory lesions in transgenic animals was variable depending on the organs to which IL-10 was targeted. The lesions were milder
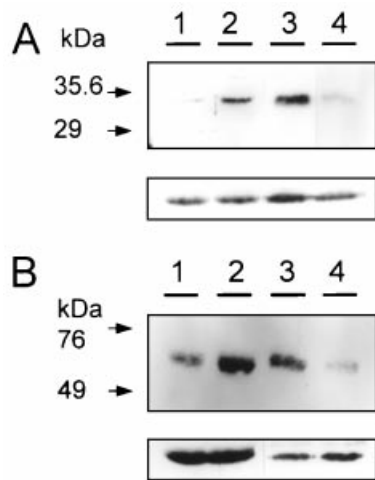
Figure 6   Western blot analysis of TRAIL and DR4 in the mammary gland

(**A**) Expression of TRAIL in the mammary gland. Upper panel: cell extracts were prepared from mammary tissues of normal mice at lactation day 8 (lane 1) and involution day 7 (lane 2), from IL-10 transgenic mice at lactation day 8 (lane 3) and from IL-10$^{-/-}$ mice at involution day 7 (lane 4). Each lane was loaded with 10 μg of protein, which was separated by SDS/PAGE [13.5% (w/v) gel]. The protein was detected with rabbit polyclonal anti-TRAIL antibody. Antibody detection was performed with the enhanced chemoluminescence (ECL) Western blot detection system (Amersham Life Science). Lower panel: control immunoblot for the mouse β-actin. (**B**) Expression of DR4 in the normal and IL-10 transgenic mouse. Upper panel: DR4 protein was assayed at lactation (lane 1) and involution (lane 2) from normal mammary glands. The protein was also assayed with the use of 1 μl of milk (1:6 dilution) from a transgenic mouse (lane 3) and from a normal mouse (lane 4). A mouse monoclonal anti-DR4 antibody was used to detect DR4. Lower panel: control immunoblot for the mouse β-actin (lanes 1 and 2) and lactoferrin (lanes 3 and 4).

when IL-10 was targeted to the mammary gland than when targeted to the salivary gland [12]. In support of this, the salivary glands in our transgenic mice developed inflammatory lesions more easily than the mammary glands, even though the expression of IL-10 was much lower in the latter (Figures 3E and 3F). These results imply that the Fas-L/Fas-mediated pathway through lymphocyte infiltration is not enough to invoke apoptosis of the cells in the mammary gland, in contrast with the salivary gland, so some other signals might be involved. The induction of IL-10 at the involution might be linked through Fas–FasL interaction with the tolerance of the immune system to prevent organ injury, as shown in the eye [32], the placenta [33] and the testis [34].

The specific increase in expression of the gene encoding TRAIL among the apoptosis-related genes by IL-10 indicates that IL-10 participates in apoptosis through a specific death pathway in the mammary gland. This is supported by the lower expression of TRAIL in the IL-10$^{-/-}$ mice. Previous studies *in vitro* have shown that TRAIL induces apoptosis in some tumour cell lines, which is mediated by DR4 and DR5 [35–37]. However, although both TRAIL and TRAIL receptors are constitutively expressed in various tissues, it was found that TRAIL might not induce apoptosis of most non-transformed cells [38]. It was suggested that unlike other members of the TNF family, TRAIL is instead a potent inhibitor of cell cycle progression in normal cells [39]. In the present study, it is suggested that IL-10 induces death receptor genes, such as DR4 via TRAIL.

Taking these studies and our results together, we speculate that IL-10 is up-regulated by specific signals in mammary epithelial cells at the involution stage; this then induces TRAIL

to inhibit the cell cycle progression of the epithelial cells at that stage. In addition, IL-10 induces a mild infiltration by lymphocytes, which could potentially induce the Fas-mediated apoptotic pathway.

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