Transcription of Granzyme A and B Genes Is Differentially Regulated during Lymphoid Ontogeny

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Summary

During development, thymocytes express a number of genes typical for activated peripheral T lymphocytes, including granzymes. We have now analyzed by reverse transcription–polymerase chain reaction (RT-PCR), immunohistochemistry, and cytochemistry fetal liver cells and thymocytes at various developmental stages for the expression of granzyme A–G genes. At days 13–17 of gestation, only granzyme B but none of the other granzymes is expressed in fetal liver. In the most immature, Pgp-1⁺ IL2Rα⁻, thymocyte subpopulation mRNAs for granzymes A–C but not for granzymes D–G are detectable. Upon further differentiation via Pgp-1⁻ IL-2Rα⁺ into more mature Pgp-1⁻ IL2Rα⁻ thymocytes the level of expression of granzymes A, B, and C gradually declines reaching its lowest level at the CD4⁺8⁺ double positive stage. In fetal thymic lobes depleted of lymphoid cells by treatment with deoxyguanosine, no transcripts for granzymes A, B, and C were found indicating that the PCR signals are derived exclusively from early precursor T/natural killer (NK) lineage cells rather than from residual stromal elements. In mature CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes, granzyme B mRNA is found at similar levels in both subsets whereas granzyme A mRNA is expressed selectively in the CD4⁺CD8⁺ subset. Enzymatic activity of granzyme A was only seen in a fraction of CD4⁻CD8⁺ thymocytes negative for heat stable antigen (HSA) but not in the more immature HSA⁺ fraction of CD4⁻CD8⁺ thymocytes. The data suggest that (a) granzyme B is a pro-thymocyte marker for all T/NK lineage cells; (b) granzyme A transcripts are associated with thymocytes with the potential to develop into the CD8⁺ lineage; and (c) granzyme A enzymatic activity is only expressed in the most mature CD4⁺CD8⁺ stage, suggesting that granzyme proteins are not involved in early stages of thymocyte development.
tion. Whereas in fetal liver only granzyme B is transcribed, early thymocyte stages contain mRNAs for granzymes A, B, and C. At the double positive (DP) stage, all granzyme transcription is very low. Either of the mature single positive (SP) subsets transcribes the granzyme B gene but granzyme A transcripts are only present in CD8+CD4⁻ cells. Enzymatic activity of granzyme A is detected only in the most mature CD8⁺CD4⁻ cells, suggesting that granzyme transcription reflects lineage potential rather than the requirement of functional gene products during development.

Materials and Methods

Mice. 13–17-d-old pregnant female BALB/c mice were obtained from the specific pathogen-free breeding facility at the Max-Planck-Institute for Immunobiology.

Monoclonal Antibodies. Anti-CD3ε antibody 145-2C11 (21), hamster IgG, was isolated from culture supernatants by affinity chromatography using a Protein A column (Pharmacia, Freiburg, Germany). For flow cytometry, labeled anti-CD8 (53.6-7), anti-CD4 (RM-4-5), anti-CD3ε (500A2), anti-Fpg-1 (IM7) (all purchased from Pharmingen, Hamburg, Germany), and fluoresceinated anti-IL-2Rα antibody SA2 (22) were used.

Fetal Thymic Organ Cultures. Fetal thymic lobes were prepared from fetuses at day 14 of gestation. They were cultured on filter discs floating on 1 ml of IMDM supplemented with 10% selected FCS, 2% glutamine, and 1% Penicillin/Streptomycin in 12-well dishes (Costar Corp., Cambridge, MA) at 37°C in 7% CO₂/air, as described by Jenkinson et al. (23). For antibody treatment, the lobes were suspended for 2 h in medium supplemented with 50 μg/ml anti-CD3 mAb and subsequently placed on filter discs floating on the same medium until analysis. Control lobes were supplemented with normal hamster IgG. Exposure to antibodies was started after 6 d of culture. Cultures were terminated after 2×10⁵ viable cells after dissociation with Trypsin/EDTA.

Flow Cytometry and Cell Sorting. Three-color staining was performed using FITC-, PE-, and biotin-labeled Ab. As a third-color Tricolor-conjugated Streptavidin (Medac, Hamburg, Germany) was used. Analytical flow cytometry was performed on a FACScan Plus® (Becton Dickinson, Heidelberg, Germany). Cell sorting was performed on a FACStar Plus® (Becton Dickinson). Cells were obtained from day 16 or 17 fetal thymuses, or from adult thymus.

RT-PCR and Southern blot Analysis. Cytoplasmic RNA was prepared using the Acid Guanidinium-Thiocyanate-Phenol extraction method (25). RT-PCR was performed essentially as described (13). Briefly, 1 μg of total RNA was subjected to RT in the presence of 20 U RTase inhibitor from human placenta (Boehringer-Mannheim, Mannheim, Germany) and 5 U of Superscript reverse transcriptase ( Gibco/ BRL, Eggenstein, Germany) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (wt/vol) gelatin, 1.5 mM MgCl₂, and 0.1% Triton X-100. Both primers were at a concentration of 1 μM, and each dNTP (dATP, dCTP, dGTP, and dTTP; Pharmacia) at 200 μM. The reaction mixture was incubated for 1 h at 42°C, then RT was stopped by heat inactivation (5 min, 95°C). After addition of 0.2 U Super-Taq-Polymerase (Steinhel, Basel, Switzerland) and overlaying with 50 μl mineral oil (Sigma, Deisenhofen, Germany) the cDNA was subjected to 35 cycles of PCR amplification as follows: denaturation for 70 s at 94°C, primer annealing for 2 min at 45°C, and primer extension for 2 min at 72°C. For Southern blot analysis, 5 μl of PCR products was resolved on a 1.5% agarose gel and blotted onto a nylon membrane by alkaline transfer in 0.5 M NaOH, 1.5 M NaCl using a vacuum blotting device (Pharmacia). Filters were hybridized to radiolabeled oligonucleotide probes at 10⁶ cpm/ml in 5× SPBR, 5× Denhardt’s solution, 0.1% SDS, 10 μg/ml salmon sperm DNA, then washed, dried, and exposed to x-ray films. The sequences of the oligonucleotides used as PCR primers and as hybridization probes as well as the sizes of the expected PCR products are described elsewhere (13). All PCR experiments were done at least in duplicates.

Analysis of BLT-esterase Activity. FACS®-sorted thymocytes were resuspended in NKH buffer (137 mM NaCl, 5.3 mM KCl, 20 mM Hepes) at 1×10⁶ cells/ml, allowed to adhere for 1 h to adhesion slides (BioRad, Muenchen, Germany), blocked with NKH, 0.2% BSA for 5 min, and finally fixed with ethanol for 5 min. To assay for BLT-esterase activity, a freshly prepared solution of 0.5 mg/ml Fast Blue BB salt, 100 μM N-o-Benzyloxy-1-Lysyl-Thiobenzyl-Ester (BT; both from Sigma) was added to the cells. After 15 min of incubation at room temperature, the cells were counterstained with hemalaun solution (Merck, Darmstadt, Germany) and inspected microscopically. The frequency of cells positive for granzyme A enzymatic activity was calculated from the analysis of 1,000 cells per thymocyte subpopulation.

Immunohistochemistry of Cytosections. 4–6 μm cytosections of fetal thymus were fixed in acetone. Endogeneous peroxidase activity was inhibited by incubating the sections for 20 min in TBS (0.1 M Tris, 0.1 mM NaCl) pH 7.6, and 0.03% H₂O₂. After blocking with TBS, 1% FCS and 2% nonfat dry milk, the sections were incubated with the primary antibodies for 1 h. The following primary antibodies were used: mouse anti–mouse CD8 biotinylated (clone 19.178, IgG2A), rat anti–mouse granzyme A (clone 7.1, IgG; 26). Subsequently, the sections were incubated with either streptavidin-peroxidase (Dianova) or goat anti–rat Ig-alkaline phosphatase for 30 or 45 min, respectively, and after washing either TBS, 0.5 mg/ml diaminobenzidine (Sigma) or 0.1 M Tris (pH 8.2), 0.5 mg/ml naphthol, 50 mM levansol, and 1 mg/ml Fast Red (all from Sigma) was added. The staining procedure was controlled microscopically and the nuclei were counterstained with Mayer’s hemalaun (Merck). At least 10 sections per fetal thymus were inspected.

Results and Discussion

Expression of Granzymes A-G in Thymocyte Subpopulations. During early T cell development, bone marrow (or fetal liver–) derived precursor cells enter the thymus where they develop into mature SP CD4⁺ or CD8⁺ T cells (27). This maturation involves a process of differential gene expression and rearrangement of the TCRα and β gene loci. The earliest precursors express Pgp-1 and FcγRII/III. They do not express CD4 or CD8 at high levels, and the TCR genes are still in germline configuration. These cells acquire the α chain of the IL-2 receptor (IL-2Rα), lose Pgp-1 and FcγRII/III, and start to rearrange the TCRβ locus. After successful rearrangement, the thymocytes express an immature TCR which drives the cells to downregulate IL-2Rα and subsequently to mature to the DP CD4⁺CD8⁺ stage. This is accompanied by a burst of cell divisions (27). Interestingly,
very early thymocytes express many proteins that are considered activation markers of mature T cells, such as CD25, intercellular adhesion molecule 1, and CD59 (19). The activation of these genes is not regulated by the immature TCR β-CD3 complex as it was also observed in Rag-2-deficient mice (19). However, it is possible that activation occurs by CD3 complexes expressed before TCR β gene rearrangement as previously demonstrated in Rag-1-deficient mice (28, 29). From earlier studies it was known that granzymes A and B are also expressed in DN thymocytes (18). By in situ hybridization it was shown that ~8% of IL-2Rα+ thymocytes, and ~50% of IL-2Rα- thymocytes express granzyme A mRNA. Very few of these immature double negative (DN) thymocytes express mRNA for granzyme B (3%). The purpose of this study was to analyze the expression patterns of all known murine granzymes (A–G) in more detail.

To this end, we sorted thymocytes from 16- or 17-d-old fetuses in Pgp-1+IL-2Rα- (Pgp-1+), Pgp-1-IL-2Rα+ (IL2Ra+), Pgp-1-IL-2Rα- (Pgp-1-), or from day 17 of gestation into double negative (DN) and double positive (DP) thymocytes. Thymocytes from adult mice were separated into TCR high and TCR low thymocytes, and the TCR high cells were further subdivided into CD4+CD8- (CD4+) and CD8+CD4- (CD8+) thymocytes. RNA from CTLL 1.3E6 (+) and from the thymoma line EL4F15 (-) was used as positive and negative control, respectively. Note that granzyme B is expressed at low levels in EL4F15 as shown previously (13). Total RNA was isolated from the sorted cells and granzymes A–G-specific mRNAs were amplified by RT-PCR. The amplification products were blotted onto nylon membranes and hybridized to 32P-labeled oligonucleotide probes specific for each of the seven granzymes. Filters were exposed for 30 min (A), 6 h (B), and 16 h (C). HPRT blots were exposed for 20 min in all cases.
enzymes A–G by RT-PCR. Sorting included gating for scatter parameters that exclude macrophage/monocytes and dendritic cells. The amount of RNA present in the reactions was standardized to equal amounts of RNA encoding hypoxanthine-guanine phosphoribosyl transferase (HPRT).

From the seven granzymes, only mRNAs for granzymes A, B, and C were detectable in various thymocyte subpopulations (Fig. 1). Highest expression of mRNA for granzymes A and B was observed in the most immature Pgp-l−II-2Rα− thymocyte subpopulation. During further maturation into Pgp-l−II-2Rα+ and Pgp-l+II-2Rα−, the mRNA levels of both granzymes decreased, and transcripts were hardly detectable in DP thymocytes (a weak signal for granzyme A was seen after exposure of 70 h, data not shown). As Pgp-l+II-2Rα+ thymocytes do not undergo functional rearrangement of the TCR genes, it can be concluded that granzymes A, B, and C are transcribed independent of TCR α/β or γ/δ gene expression in this thymocyte subpopulation. The possibility that granzyme gene transcription is merely correlated with the proliferation status of the thymocytes is unlikely since large DP thymocytes are rapidly cycling, but contain low amounts of granzyme A and B and no granzyme C–G transcripts.

To exclude the possibility that the PCR signals for granzymes A, B, and C are derived from a minor contaminating population of non-T/NK lineage thymocytes, thymic lobes from day 14 of gestation were either cultured alone (Thy−) or in the presence of deoxyguanosine (Thy+) cells from untreated thymic lobes and from thymic rudiments were analyzed for the presence of granzyme A, B, and C-specific mRNA by RT-PCR, as described in the legend to Fig. 1. The blots were exposed for 16 h. HPRT transcripts for granzymes D–G were not detectable. Together with the previous finding that in the periphery granzyme A is expressed in the majority of CD8+ but only in a fraction of CD4+ cells (32), the data suggest that the expression pattern of granzyme A in the thymus reflects functional commitment to the cytotoxic lineage.

**Granzyme A Protein Expression.** To further analyze granzyme A mRNA and protein expression in the CD8+ SP thymocyte subpopulation, TCRhiCD8+ SP thymocytes were separated into two subpopulations distinguished by the expression of heat stable antigen (HSA), i.e., TCRhiCD8+HSA+ and TCRhiCD8+HSA−. HSA was shown before to be highly expressed on DP and early SP cells, but much less on mature thymocytes and peripheral T cells (33).

Both subpopulations contained similar amounts of granzyme A mRNA as analyzed by RT-PCR (Fig. 3 A). However, granzyme A proteolytic activity was only detected in the HSA− but not HSA+ subset (Fig. 3 B) indicating that granzyme A is expressed at the protein level only in the most mature CD8+ SP thymocyte subset. Since HSA− and HSA+ SP cells expressed about equal amounts of granzyme A mRNA, it is unlikely that the differential expression of enzymatic activity is due to limits in the sensitivity of the assay. The frequency of cells containing granzyme A activity was ~1% which corresponds to the frequency of granzyme A mRNA expressing CD4+CD8+CD3+ thymocytes as detected by in situ hybridization (18). No granzyme A enzymatic activity was found in any of the more immature thymocyte subpopulations tested (data not shown).

In addition, cryosections of adult thymus were stained with a granzyme A-specific mAb (Fig. 3 C). Only few scattered cells positive for granzyme A were seen which were mainly associated with the medulla. No staining was seen in the cortex harboring the majority of immature thymocytes. Taken together, these findings indicate that granzyme A mRNA is expressed at various stages during thymocyte development but is translated into protein only in the most mature CD8+ SP subpopulation.
Figure 3. Analysis of granzyme A protein expression. CD8+CD4- HSA+ and CD8+CD4-HSA- thymocytes from adult mice were analyzed for the presence of granzyme A-specific mRNA by RT-PCR (A; 16-h exposure), or for the presence of granzyme A enzymatic activity by BLT-esterase assay after fixation on adhesion slides (B; × 1,000). Cryosections of adult BALB/c thymus were stained with an antigranzyme A mAb (C; × 400).
of granzymes in mature cells is restricted to T and NK cells (13, 15, 30). Therefore, it is likely that the early DN thymocytes transcribing granzyme A-C genes are already committed to these lineages. To test the possibility that commitment to the T/NK lineage already takes place in the fetal liver, we analyzed RNA from fetal liver from fetuses at days 13–17 of gestation for the presence of transcripts for granzymes A–G. As shown in Fig. 4, granzyme B mRNA is detectable in fetal liver at all days of gestation tested with slight increases in mRNA during the development of the fetuses (at days 13–15 granzyme B–specific transcripts were detectable only after 72-h exposure, data not shown). No mRNAs for granzymes A or C–G were detected even after prolonged exposure. At present it is unclear whether among granzymes A–G the granzyme B gene is transcribed selectively during these early stages of development or whether the frequency of cells transcribing granzyme genes is very low and granzyme B mRNA is more abundant than mRNAs derived from the other granzymes. Since expression of granzymes so far has been described to be restricted to hematopoietic cells of lymphoid origin and within those to T and NK cells (34–36), our findings support the hypothesis that the fetal liver already contains precursor cells committed to the T/NK lineage. NK precursor activity has been described before in fetal liver cells, which, however, was only detectable upon their in vitro stimulation with IL-2 (37). Granzyme B may therefore be a useful marker for the detection of T/NK committed precursor cells in early stages of gestation.

**Granzyme Expression in Fetal Thymic Organ Cultures.** Since granzymes A and B are known to be involved in DNA fragmentation of target cells (7–10) it was possible that these proteinases play a role during negative selection leading to depletion of autoreactive thymocytes at the DP stage. According to our first experiments (Fig. 1), expression of granzymes A and B is particularly low at that stage compared to more immature thymocyte subpopulations. To test the possibility that granzymes A and B or any of the other granzymes are induced during negative selection, fetal thymic lobes from day 14 of gestation were cultured on filter discs for 6 d, and then incubated with anti-CD3 mAbs for an additional 3, 5, and 8 h. Subsequently, the lobes were analyzed for expression of granzymes A–G by RT-PCR.

Under these conditions only transcripts for granzymes A, B, and C but none for any of the other granzymes are found (Fig. 5). Comparing the anti-CD3–treated lobes to the control lobes no significant differences are observed. The number of DP thymocytes was reduced in the presence of anti-CD3 mAbs from 69% in control lobes to <58% in anti-CD3–treated lobes as measured by FACS® analysis (data not shown). Furthermore, DNA fragmentation was induced by

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**Figure 4.** Analysis of expression of granzymes A–G in fetal liver. Fetal liver was isolated from embryos at day 13–17 of gestation and total RNA from the liver cells was analyzed for the presence of granzymes A–G–specific transcripts by RT-PCR as described in the legend to Fig. 1. The blots were exposed for 30 min (A) and 16 h (B). HPRT blots were exposed for 20 min.
the anti-CD3 treatment as measured by agarose gel electrophoresis (data not shown), indicating that negative selection occurs under these conditions.

These findings indicate that granzyme mRNA expression is not upregulated during negative selection and argue against the involvement of these proteinases in programmed cell death during negative selection of thymocytes. This assumption is in line with the observation that thymocytes from granzyme B-/- mice undergo normal programmed cell death in response to dexamethasone or staphylococcal enterotoxin B stimulation (10) and that granzyme A-deficient (Ebnet, K., M. Hausmann, F. Lehmann-Grube, J. Golecki, M. Kopf, M. C. Lamers, and M. M. Simon, manuscript in preparation) as well as granzyme B-deficient mice (10) show unaltered hematopoiesis and development of peripheral lymphoid cells.

Conclusion. Taken together, our findings show that (a) in fetal liver granzyme B transcripts are detectable at days 13-17 of gestation; (b) in the most immature, Pgp-1+IL-2Rα−, thymocyte subpopulation the genes for granzymes A, B, and C are transcribed; (c) from the two mature thymocyte subpopulations, only CD4+CD8− but not CD4+CD8+ cells express granzyme A; and (d) granzyme A protein is expressed only by the most mature CD4+CD8− thymocytes.

The finding that fetal liver cells express transcripts for granzyme B, a marker for antigen-activated T cells and mature NK cells, suggests that the commitment of hematopoietic cells to the T/NK lineage already occurs at this stage of development. Granzyme B might represent a marker for these lineages suitable to study the differentiation pathways of early precursors of NK and T cells.

The presence of granzyme A, B, and C transcripts in early fetal thymocytes is reminiscent of other markers which are associated with activated peripheral T cells but temporarily induced in immature TCRβ thymocytes (19). It seems very likely that the fetal liver/thymic stroma tissues provide the activation signals required for the induction or maintenance of granzyme A, B, and C mRNA expression. It remains to be determined if the immature CD3 complex expressed before TCRβ rearrangement (28, 29) is involved in this induction event.

The functional relevance of granzymes during T/NK cell development is not known. The presence of mRNAs does not necessarily imply the presence of functional proteins and our findings indicate that only the most mature, i.e., CD4+CD8+HSA−, thymocytes express granzyme A as protein. The presence of granzyme transcripts in early thymocytes may reflect an activation state necessary to start transcription and rearrangement of the TCR gene loci. It is unlikely that the functional expression of the granzyme proteins is relevant for T cell maturation. This is supported by the normal development of T cells in mice lacking granzyme A (Ebnet, K., et al., manuscript in preparation) or granzyme B (10). In more mature cells, transcription of genes for granzymes A and B is most probably correlated with the functional commitment to the CD4+ and CD8+ lineages. Because gran-
zyme A is expressed in peripheral CD8⁺ T cells only upon activation (38), the expression in CD8⁺ thymocytes may correlate with a phase of activation necessary for peripheral expansion of newly produced T cells. Alternatively, positive selection itself may be accompanied by a state of activation, as suggested by expression of activation markers such as CD69 (39).

From the seven granzymes A-G described so far in the mouse, only granzymes A, B, and C are transcribed during early hematopoiesis. The same expression pattern of granzymes is observed in in vivo activated peripheral T cells (13). Transcription of granzyme D–G genes was detectable so far only after short-term in vitro culture (14, 15). The stimuli required for the induction of granzymes D–G in vivo as well as their physiological relevance are not known.

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