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Reduced expression of CD99 and functional disturbance in anencephalic cortical thymocytes

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Abstract In a significant proportion of cases, anencephaly is associated with thymic enlargement, suggesting a possibility that anencephalic fetuses have a functional disturbance in thymocyte differentiation and development. In this report, we demonstrated that CD99 expression was consistently reduced in cortical thymocytes of all anencephalic fetuses. In addition, the CD99-dependent aggregation of immature cortical thymocytes was almost completely impaired and apoptosis of thymocytes was markedly reduced in several cases. These results are in agreement with previous findings that CD99 regulates the aggregation and apoptosis of various types of cells. These data strongly suggest that functional disturbance of thymocytes and thymic hyperplasia are related to the reduced expression of CD99 molecule in anencephalic fetuses.

Key words Anencephaly · Thymic hyperplasia · CD99 · Apoptosis · Aggregation

Introduction

Anencephaly is among the most common major congenital malformations, with an estimated incidence of 1 in 1,000 births [20]. It is divided into two subtypes: meroanencephaly (the presence of rudimentary brain tissues and partial formation of the cranium) and holoanencephaly (no cranial vault) [18]; both result from a failure of neural tube closure. This failure appears to be multifactorial, arising from the combined effects of several genetic abnormalities and environmental hazards [16].

Other anomalies are frequently observed in anencephalic fetuses, including hypoplastic adrenal glands and a small anterior pituitary gland with no posterior lobe [23]. The thymuses of anencephalic fetuses have been shown to be hyperplastic [1], and thymuses in decapitated rat fetuses also show hyperplasia [3]. It has been reported that the mean weight of thymuses in newborn anencephalic infants is greater than that of control infants, and several lines of evidence support an association of thymic hyperplasia with anencephaly [2, 8, 12, 22]. However, the cause of thymic hyperplasia in anencephalics is not clear.

CD99 is a transmembrane sialoglycoprotein encoded by the pseudoautosomal gene *mic2*, which is homologous with Xga (PBDX) and escapes X chromosome inactivation [11, 29]. It is distributed in a wide range of human tissues such as haematopoietic cells, pancreatic islet cells, granulosa cells of the ovary, Leydig and Sertoli cells of the testes and ependyma, and some malignant cells [10]. Despite its wide distribution, the expression levels of CD99 vary and are highly dependent on cell type. Although the function of CD99 is unclear, it is evident that engagement of CD99 induces homotypic aggregation and apoptosis of CD4+ CD8+ human thymocytes [4, 5, 13] and of other types of cell, such as RD-ES, an Ewing's sarcoma cell line [30]. We have recently shown that CD99 regulates the expression of several thymocyte surface molecules, such as TCR and MHC I and II, and suggested that it is implicated in the development and differentiation of human thymocytes [9].

This report deals with the pattern of CD99 expression, the functional response of anencephalic thymocytes to CD99 engagement, and the extent of apoptosis in anencephalic thymuses. CD99 expression in anencephalic thymocytes was significantly reduced, and subsequently,

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Table 1 Levels of CD99 expression and apoptosis in thymuses of anencephalic and control fetuses (*GA* gestational age, *ND* not determined; CD99: – negative, + weakly positive, ++ strongly positive; apoptosis: – not detected, ± less than 2, + 2–15, ++ more than 15 nuclei stained. at ×600)

GA (weeks)	Control fetuses			Anencephalic fetuses		
	Case	CD99	Apoptosis	Case	CD99	Apoptosis
15				A1	–	+
16	C1	++	+			
18				A2	–	ND
19	C2	++	ND			
22	C3	++	ND	A3	–	–
23	C4	++	+	A4[a]	–	±
24				A5[b]	–	+
24				A6[a, b]	–	±
25	C5	++	ND			
27				A7	–	ND
28	C6	++	++			
29				A8	–	+
33	C7	++	+	A9	–	±

[a] Meroanencephaly
[b] Hyperplastic thymus

functional derangements in apoptosis and CD99-induced aggregation were also observed. On the basis of these findings, we suggest that thymic hyperplasia and functional disturbances in anencephalic fetuses are associated with the down-regulation of CD99 expression in their thymocytes.

Materials and methods

Sixteen autopsy cases, including 9 of anencephaly, originally reported between 1991 and 1996 were selected from the archives of the Department of Pathology at Seoul National University Children's Hospital, Chungbuk University Hospital, and Dong Kook University Hospital. The diagnoses had all been confirmed by retrieval of pathology reports. All tissues had been fixed in 10% neutral-buffered formalin and embedded in paraffin. In addition, two anencephalic fetuses and two control fetuses were obtained within 24 h of legal termination of pregnancies. All related persons gave their informed consent prior to inclusion of their fetuses in this study. The gestational age of each fetus was retrieved from maternal records and ranged from 15 to 33 weeks (Table 1). The clinical details of the control fetuses are given in the Table 2.

Table 2 Clinical details of control fetuses

Case	Malformation	Cause of death
C1	Amniotic band syndrome	Fetal death in utero
C2	Gastroschisis	Therapeutic termination
C3	Anencephaly	Therapeutic termination
C4	Thanatophoric dysplasia	Therapeutic termination
C5	Potter syndrome	Therapeutic termination
C6	Potter syndrome	Therapeutic termination
C7	Achondrogenesis type II	Therapeutic termination

Anti-human CD99 (DN16) and anti-human CD46 (AP15) monoclonal antibodies (mAbs) were obtained from hybridoma clones developed in our laboratory [9, 13, 30]. DN16 and AP15 mAbs stain all human thymocytes on flow cytometric analysis. Since DN16 mAb induces the aggregation and apoptosis of human thymocytes, and easily detects CD99 on the surface of all T cells, it is evident that epitope defined by DN16 can be categorized into those recognized by O662 and those recognized by L129 [4, 5, 9, 13]. Another anti-human CD99 mAb (12E7), which recognizes CD99 on the surface of T cell subsets, was purchased from DAKO (Carpinteria, USA) [5, 9, 13].

Paraffin sections from 16 cases were processed for CD99 immunohistochemistry. The sections were deparaffinized in xylene and hydrated through ethanol to phosphate buffer (0.1 M, pH 7.3); this was followed by rinsing in 3% H_2O_2 to remove endogenous peroxidase activity, and in 10% (vol/vol) normal goat serum to reduce nonspecific binding of immunoglobulins. The sections were incubated with mouse anti-CD99 and anti-AP15 mAbs in a humid chamber for 2 h at room temperature. After rinsing with phosphate-buffered saline (PBS), the sections were incubated for 30 min at room temperature with affinity-purified biotinylated goat anti-mouse immunoglobulin G (IgG), and then in a 1:1,000 dilution of streptavidin–horseradish peroxidase conjugate (DAKO). The chromogen was developed for 2 min with 0.5 mg/ml 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, Mo.) in the presence of 0.3% H_2O_2, and brown precipitates, an indication of the presence of antigen–antibody–horseradish peroxidase complexes became obvious under a light microscope. The concentration of the DAB–H_2O_2 mixture must be adjusted and the developing time for each section should be strictly controlled, since they are crucial in quantitative immunohistochemistry, as for any biochemical enzymatic assay, to make proper visualization of comparable colouration of different expression levels of antigen possible [26]. Counterstaining was not performed, since the staining pattern of the serial sections with haematoxylin-eosin was available. The photographs of anencephalic and control thymuses were taken under the light microscope (original magnification, ×200).

Two anencephalic and two age-matched fetal thymic tissues were obtained from fetuses prepared for autopsy after legal termination of pregnancies. Within 24 h, fresh thymic tissues were minced with sharp scissors in RPMI 1640 media after careful removal of blood clots and fibrous capsules. Viable thymocytes were isolated by Ficoll-Hypaque density centrifugation and washed twice in RPMI media, and fresh thymocytes were resuspended in PBS containing 0.05% sodium azide and 0.2% bovine serum albumin. One million thymocytes were incubated with primary antibodies at 4°C for 30 min, washed three times, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zymed Laboratories, Calif.) at 4°C for 20 min. Negative control samples were incubated with isotype-matched control antibody and subsequently with FITC-conjugated secondary antibody. The stained cells were analysed with a FACScan® (Becton Dickinson, Calif.).

Paraffin sections of fetal thymuses were deparaffinized, rehydrated, treated with proteinase K (20 µg/ml), and blocked for endogenous peroxidase activity with 3% H_2O_2 for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay. Subsequent end-labelling with TdT (0.3 U/µl) in TdT buffer together with 2 µM biotinylated 16-dUTP was carried out for 1 h at 37°C in a humidifying chamber. After the end-labelling, the sections were washed in PBS and incubated with streptavidin-conjugated horseradish peroxidase complex, rinsed in PBS, and stained with diaminobenzidine. The slides were washed, dried, and

mounted in Permount medium. The photographs of anencephalic and control thymuses were taken under the light microscope (original magnification, ×40 and ×400). Since no apoptosis was observed at the thymic medulla, several cortical areas of at least three different sections for each thymic specimen were chosen at random, and cell counting was performed at ×600 magnification. The apototic nuclei were identified by the presence of dark brown staining.

Resuspended thymocytes (5.0×10^5 cells/well) were plated in flat-bottomed 96-well microtitre plates. DN16 (10 µl, 100 µg/ml) was added and crosslinked by goat anti-mouse IgG (500 µg/ml). Cells were incubated for 4 h at 37°C, and the degree of aggregation was measured at the indicated time points. Semiquantitative scoring of the index of adhesion was conducted as follows [4, 13]: 0, no adhesion or cell cluster formation (>90% of the cells were unaggregated); 1, the majority of cells were unaggregated, but a few small clusters of <20 cells were observed (this level of adhesion is typical of the spontaneous adhesion exhibited by many lymphoblastoid cell lines); 2, 50% of the cells were in medium-sized aggregates (20–50 cells), with the remainder as single cells; 3, nearly all cells were in medium-sized to large aggregates (>50 cells) with only a few (<20%) unaggregated cells; and 4, >90% of the cells were in large aggregates. After incubation at 37°C for 4 h, photographs of the treated cells were taken under the light microscope (original magnification, ×40).

Results

The gestational age of each of the 16 cases is shown in Table 1. With reference to organ weight data on normal Korean fetuses [7], organ weights of anencephalic fetuses above 19 weeks of gestation were compared with the mean values±standard deviation of age-matched organ weights. As previously reported [2, 3, 8], adrenal glands in anencephaly were clearly hypoplastic, and this condition became much more obvious as gestational age increased (data not shown). Although adrenal hypoplasia was observed in all anencephalics, definite thymic hyperplasia was found in only two anencephalics (A5 and A6; data not shown).

In order to evaluate the expression pattern of CD99 in the thymus, thymic tissue sections of anencephalic and age-matched control fetuses were immunostained with anti-CD99 mAb (DN16 and 12E7) or an isotype-matched control (anti-CD46 mAb; AP15). CD99 was found to be highly expressed in the cortex of all control

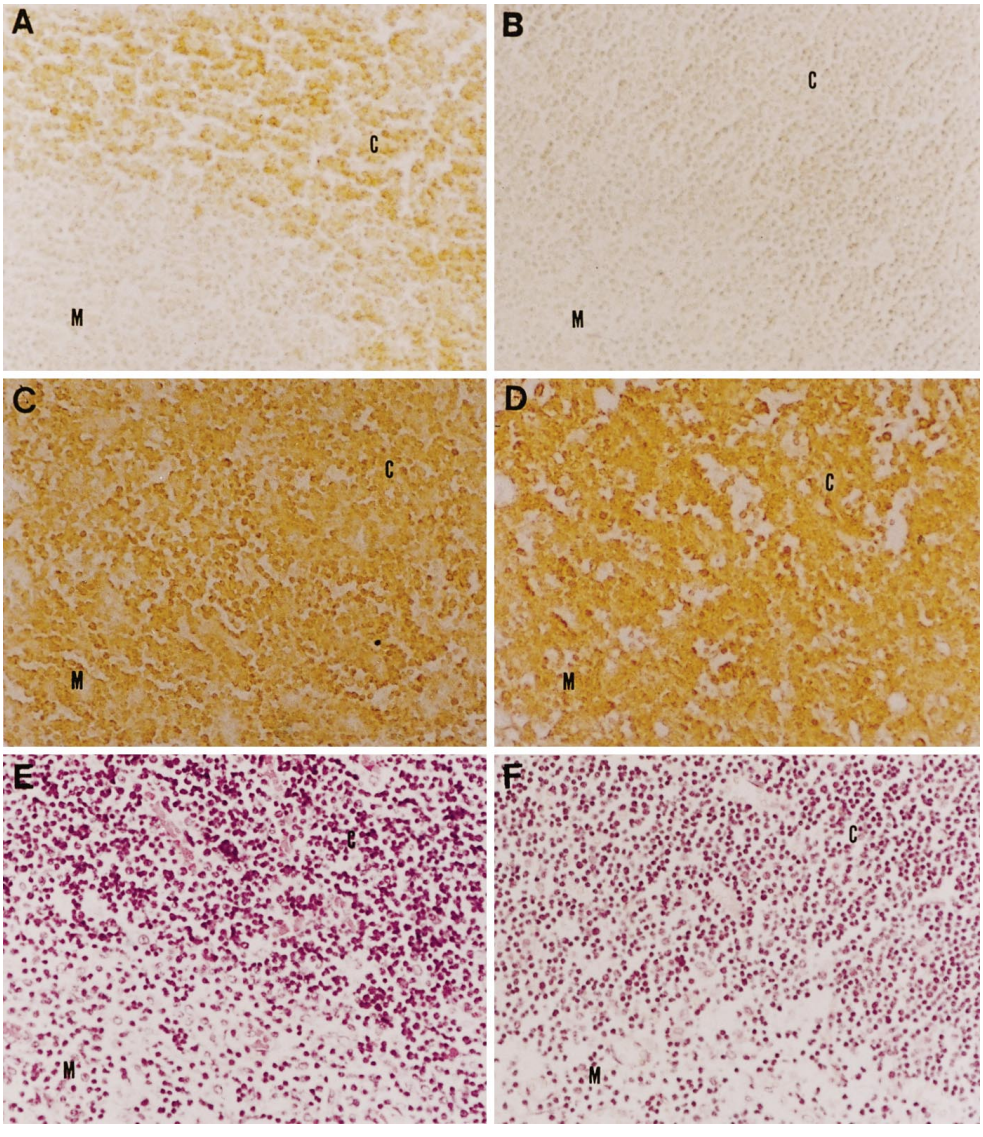

Fig. 1A–F CD99 immunostaining of the thymuses from an anencephalic and a control fetus. A control thymus (**A**, **C**, **E**) and an anencephalic thymus (**B**, **D**, **F**) at 33 weeks of gestation were immunostained. **A**, **B** Immunostaining for CD99; **C**, **D** Immunostaining for CD46; **E**, **F** H&E. Note the lower expression of CD99 in thymic sections from the anencephalic fetus than in those from the control fetus

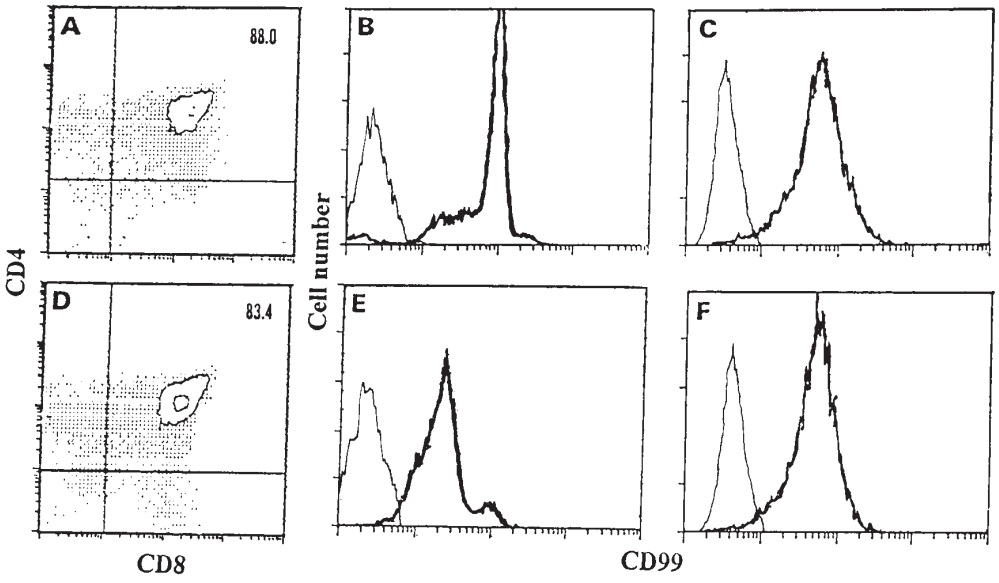

Fig. 2A–F Flow cytometric analysis in thymocytes and lung fibroblasts from an anencephalic fetus at 16 weeks of gestation and from a control fetus. Control thymocytes were surface-stained with anti-CD8-FITC and anti-CD4-PE (**A**), and anti-CD99-FITC (**B**). Anencephalic thymocytes were surface-stained with anti-CD8-FITC and anti-CD4-PE (**D**), and anti-CD99-FITC (**E**). Control lung fibroblasts (**C**) and anencephalic lung fibroblasts (**F**) were stained with anti-CD99-FITC. The *thin* and *thick lines* indicate isotype-matched controls and CD99, respectively

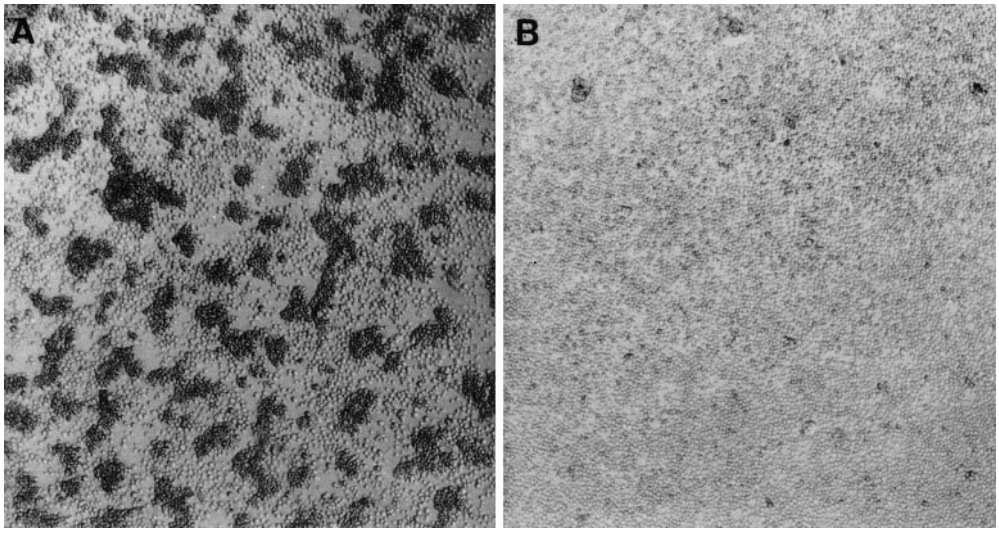

Fig. 3 Comparison of aggregation in **A** control and **B** anencephalic thymocytes by anti-CD99 mAb. Note that CD99 ligation did not induce homotypic aggregation of anencephalic thymocytes

thymuses, showing a strong diffuse membranous pattern, with clear demarcation between the thymic cortex and medulla (Fig. 1A, E). In contrast, CD99 expression was drastically reduced in all anencephalic thymuses, resulting in a loss of clear demarcation between the cortex and the medulla, (Fig. 1B, F) irrespective of subtypes of anencephaly such as holoanencephaly and meroanencephaly (Table 1). The expression level of CD46 in thymuses of anencephalic fetuses was similar to that in the control group (Fig. 1C, D).

The reduction of CD99 expression seen on immunohistochemical analysis was confirmed by comparative flow cytometric analysis of CD99 expressed on the surfaces of thymocytes and lung fibroblasts, comparing a fresh anencephalic and a normal fetus. Thymocytes from these two types of fetus showed a marked difference in their levels of CD99 expression. Control thymocytes, which were mostly cortical CD4+ CD8+ double positive (DP), expressed a high level of CD99 (Fig. 2A, B). While the majority of anencephalic thymocytes were indeed immature DP, and their CD4 and CD8 expression were similar to those of control thymocytes (Fig. 2D), marked diminution of CD99 expression was observed in DP thymocytes of anencephalic fetuses (Fig. 2E), again confirming the immunohistochemical data. In contrast, lung fibroblasts from anencephalic fetuses showed similar levels of CD99 expression to those from control fetuses (Fig. 2C, F).

We previously reported the establishment of a mouse hybridoma clone producing anti-human CD99 mAb (DN16), which induced homotypic aggregation of human thymocytes when added to culture media [9, 13]. In a recent study, we also demonstrated that homotypic aggregation after CD99 engagement is induced by up-regulation of diverse cell surface molecules, particularly cell adhesion molecules, thereby adjusting the functional

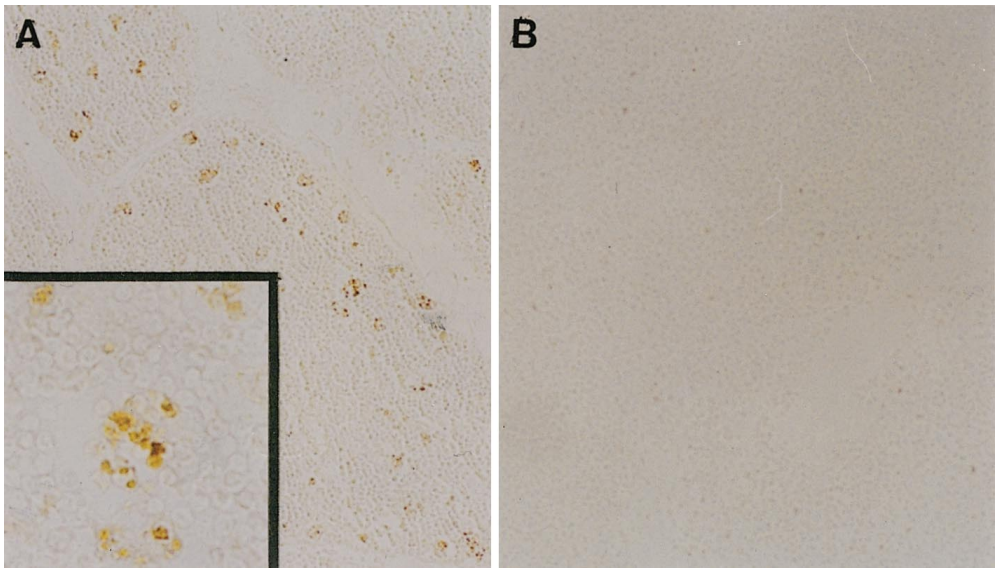

Fig. 4 TUNEL staining of **A** control and **B** anencephalic thymus. TUNEL-+ cells were hardly detected in thymic cortex of anencephalic fetus

properties of immature cortical thymocytes [9]. The kinetics of homotypic aggregation may thus be used as an index for evaluating the functional properties of thymocytes in anencephalic fetuses. To see whether any functional disturbance appeared to be related to the decreased expression of CD99 in anencephalic thymocytes, we cross-linked the CD99 molecules on anencephalic and control thymocytes with DN16 mAb and goat anti-mouse IgG as a secondary Ab. After CD99 engagement, the extent of aggregation was scored semiquantitatively on a scale from 0 to 4. In control thymocytes, the treatment of DN16 mAb induced severe homotypic aggregation of >90% of normal thymocytes in less than 4 h (score 4, Fig. 3A). In contrast, homotypic aggregation did not occur in thymocytes from anencephalic fetuses (score 0, Fig. 3B) even after 12 h of antibody treatment.

This impairment of cell aggregation in response to anti-CD99 mAb treatment in anencephalic thymocytes suggests that there is functional derangement owing to the reduction in CD99 expression in anencephalics. Because CD99 engagement induces apoptosis in immature cortical thymocytes [5], investigations of this functional disturbance have addressed the possibility of defective apoptosis of immature thymocytes. It is conceivable that defective apoptosis attributable to down-regulation of CD99 in immature thymocytes might lead to thymic hyperplasia in anencephalic fetuses.

TUNEL can be used to detect the very early stages of apoptosis where nuclei and nuclear fragments are stained, with typical morphological features of apoptotic cells. It has been reported that CD99-induced apoptosis displays a faint but significant positivity in TUNEL assay, comparable to typical apoptosis but without DNA ladder formation on gel electrophoresis [4, 30]. In order to investigate whether the overall extent of apoptosis is lowered in the anencephalic thymus compared with the normal thymus, TUNEL assays were performed. In control thymuses, the TUNEL-positive (TUNEL-+) thymocytes were observed mainly in the cortex, with negligible background staining in the live TUNEL-negative cells (Fig. 4A); this is consistent with the previous observation that apoptotic thymocytes are $CD3^{-}$ or $CD3^{lo}$ immature cells located in the cortex [31, 32]. The nuclear regions of apoptotic thymocytes were positively stained, as was the cytoplasm of some phagocytosing macrophages. It is likely that these macrophages had phagocytosed neighbouring TUNEL-+ cells, as reported previously [21, 28]. Interestingly, TUNEL-+ cells were hardly detected in the thymic cortex of the anencephalic fetus (Fig. 4B). Quantification of the number of apoptotic cells in a high-power field (×600) revealed that apoptosis of thymocytes was observed at a lower frequency in 4 of 7 anencephalic fetuses (A3, A4, A6, A9) tested (Table 1, Fig. 5).

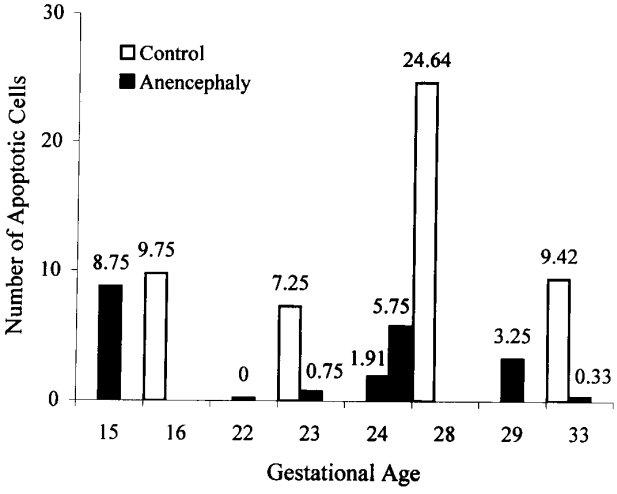

Fig. 5 Quantitation of apoptosis in anencephalic thymuses. The level of apoptosis of thymocytes was significantly lower in four of seven tested anencephalic fetuses (*A3, A4, A6, A9*)

Discussion

Many pathologists have reported that thymic hyperplasia occurs in anencephalic fetuses [1, 2, 8, 12, 22, 23]. We investigated the phenotypical and functional characteristics of thymocytes from anencephalic fetuses, including the level of CD99 expression, the frequency of apoptotic cells, and the aggregation ability in the response to anti-CD99 antibody treatment. Firstly, our data clearly indicate that the low level of CD99 expression in the anencephalic thymus is due to the reduced expression of CD99 molecules in cortical thymocytes and not to the expansion of $CD99^{lo}$ medullary thymocytes. Although CD99 molecules display isomorphism, the possibility that cortical thymocytes express the wrong isoform in anencephalic fetuses can be excluded, as we examined the level of CD99 by means of two different anti-CD99 mAbs, 12E7 and DN16 [4, 13]. Secondly, apoptosis and CD99-dependent homotypic aggregation are functionally disturbed in thymocytes from anencephalic fetuses. It has already been documented that the CD99 signaling pathway can induce diverse functional properties, such as homotypic aggregation, the up-regulation of several surface molecules, and the apoptosis of cortical thymocytes in vitro [4, 5, 9, 13]. On the basis of these data, we suggest that the reduced expression of CD99 in anencephalic thymocytes might cause functional impairment in aggregation and surface molecule expression, as well as apoptosis of cortical thymocytes, and eventually lead to defective intrathymic maturation and thymic hyperplasia. In addition, the expression of many other thymocyte surface molecules, such as JL-1 [25], CD43 and CD45 (unpublished data), was also lower in anencephalic fetuses.

The mechanism responsible for this reduced expression of CD99 is currently unclear. Two possible explanations can be suggested. The phenomenon may be associated with abnormalities of neuroendocrine–thymus axis development occurring in anencephaly, a possibility supported by the occurrence of thymic hyperplasia in decapitated rat fetuses. Although thymic hyperplasia is always accompanied by adrenal hypoplasia in decapitated rat fetuses [3], this is not likely to be due to adrenal hypoplasia, since the level of unbound glucocorticoids in normal rat plasma at birth is too low to trigger apoptosis of thymocytes [15]. Before they are 3 weeks old, adrenalectomy in rats has no effect on the thymus [17]. Rather, thymic hyperplasia is probably attributable to dysfunction of the neuroendocrine-thymus axis, which regulates thymocyte development.

CD99 down-regulation may be generated by germline mutations in some genes regulating CD99 expression or the promoter region of the CD99 gene itself, and may lead to anencephaly. Although some mouse mutants, such as MacMARCKS and Cart1 knockout mice, have shown anencephalic features [6, 14, 35], consistent gene defects associated with human anencephalics have yet to be reported. Interestingly, CD99 is located in Xp23, and terminal deletion of the Xp22 region was reported in some cases of anencephaly [27], suggesting that deletion of CD99 might be related to the pathogenesis of the defect.

Only two of our anencephalic cases had hyperplastic thymuses, but the other anencephalics have had thymic hyperplasia to some degree. Our cases were too young to show morphological changes in the thymus; most previous studies on thymic hyperplasia in anencephaly have been done with near full-term anencephalic fetuses or infants [1, 2, 8, 12, 22]. Because the thymic weight in normal fetuses varies over a wide range, it is difficult to confirm the presence of thymic enlargement in early fetuses simply by measuring the thymic weight. In our cases, thymic enlargement might therefore have been evident if they had survived and continued to grow. We expect that the decreased apoptosis in anencephalics' thymocytes may eventually lead to thymic enlargement.

The death of cortical thymocytes is primarily a reflection of apoptosis by neglect, implicating a lack of positive selection [31, 32], and it has been argued that this apoptosis probably occurs through exposure to endogenous glucocorticoids [19, 24, 33, 34]. However, hyporesponsiveness of the thymocytes to glucocorticoids in the targetted glucocorticoid receptor antisense transgenic mouse does not lead to the formation of a hyperplastic thymus [19]. Furthermore, we suggest that the apoptosis of cortical thymocytes in vivo would be more related to CD99 than glucocorticoids and that thymic hyperplasia in anencephaly should be attributed to the reduced expression of the CD99.

Our data suggest that reduced expression of CD99 is associated with functional and morphological disturbances in the thymus of anencephalic fetuses, and this paper has dealt with the possibilities of neuroendocrine regulation and genetic impairment of CD99 expression. Although the mechanism of this reduced expression is unclear, further investigation of this process may provide insights into the pathogenesis of anencephaly and functional abnormalities in other organs.

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