Ultrastructural alterations of the cortical epithelial cells of the irradiated and recovering rat thymus

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Summary. To understand the roles of cortical thymic epithelial cells (CTECs) in T-lymphocyte development, we analyzed rat thymi recovering from irradiation (6Gy), at the ultrastructural level. The morphological alterations in the CTECs were most prominent during the third to fifth day of recovery, when proliferating thymocytes were observed in the vicinity of the CTECs. The most striking finding among the alterations in the CTECs after irradiation was a cytoplasmic vacuolization with an increased amount of granular and membranous content. The granular content was observed as loosely aggregated structures or finely dispersed granules and dense bodies. The membranous content appeared in various forms including vesicular, tubular, and irregular membranous structures and myelin figures. The above features are characteristic of the hyperfunctional state of CTECs with increased secretion activities, which suggests their important roles in the repopulation and maturation of the cortical thymocytes during recovery after irradiation.

Introduction

The thymus provides a microenvironment for the development of immunocompetent T cells (Anderson, 2000). Thymic epithelial cells (TECs) appear to be a

major microenvironmental component since they produce humoral factors that directly influence the proliferation and differentiation of T cells (Van Ewijk, 1991). In addition, the major histocompatibility complex determinants on TECs are involved in thymocyte maturation (Anderson and Jenkinson, 2001; Gill *et al.*, 2003). There are two general types of TECs: cortical and medullary. In the cortex, four subtypes have been further described ultrastructurally: subcapsular/perivascular, pale, intermediate, and dark. These subtypes are also present in the medulla, but two additional TEC subtypes are also restricted to this region: undifferentiated and cystic (De Waal and Rademakers, 1997).

Irradiation causes the rapid depletion of lymphocytes in the thymus, but these lymphocytes are promptly restored by the proliferation of intrathymic and circulating bone marrow precursor cells (Kadish and Basch, 1975). We have performed a number of morphological and functional studies on the irradiated and recovering rat thymus as this regenerating thymic model provides an opportunity to examine the sequential development of immature thymocytes in adult animals: Initially, we have semiquantitatively analysed the thymocytes and stromal cell changes (mainly epithelial cell subtypes, macrophages, dendritic cells) in the recovering rat thymus after irradiation (6 Gy) (Arudchelvan *et al.*, 2000); we have subsequently done immunohistochemical studies on the irradiated (6 Gy) and recovering rat thymus at the light-microscopic level (Wang *et al.*, 1999; Mizutani *et al.*, 2002); we have also studied the cytokine message in the recovering thymus (Mizutani *et al.*, 2002; Adachi *et al.*, 2005).

Since irradiation causes the depletion of thymocytes mainly in the cortical area, it is also important to study the ultrastructure of cortical TECs (CTECs), the main cells that form the cortical microenvironment. Hypothetically:1) irradiation may produce some effects on CTECs which can

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be demonstrated at the ultrastructural level although CTECs have been believed to be resistant to irradiational changes at 6 Gy at the light microscopic level (Wang *et al.*, 1999); and 2) irradiation may cause CTECs to exhibit some features that influence the proliferation and maturation of the cortical thymocytes during recovery from irradiation. Thus, the present study was performed to investigate alterations in the ultrastructure of CTECs after irradiation.

Materials and Methods

Animals

Female DA-strain (RT-1a) rats, 9 weeks of age, were used. All animals were bred and maintained conventionally.

Irradiation procedure

The rats received whole-body irradiation (6 Gy) using an X-ray irradiator (MBR-1520R, Hitachi Medical Co. Ltd., Tokyo) operated at the rate of 1 Gy/min, with 0.5 mm aluminium and 0.1 mm copper filters. The day of irradiation was taken as day 0 of the experiment. Three rats each were killed by diethyl ether inhalation on days 0 (control), 3, 5, and 7 after irradiation.

Tissue processing for electron microscopy

Pieces of thymic tissue were quickly immersed in a solution of 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) and cut into 1 mm cubes. Following this, the tissue was fixed for 2 h in the same fixative at 4°C. The tissue was then thoroughly washed three times in a 0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide in a 0.1 M sodium cacodylate buffer at 4°C. After repeated washings, the tissue was dehydrated in a graded series of alcohol and embedded in Epon 812. The blocks were cut on a microtome (LKB- 8800 Ultratome). Semithin sections,

1 μ m thick, were routinely stained with methylene blue for light microscopy. Ultrathin sections, 60–80 nm thick, were contrasted with uranyl acetate and lead citrate, and examined with a 200 CX transmission electron microscope (JEOL, Tokyo) operating at 160 kV.

Quantitative electron microscopy

Ten micrographs (magnification $\times 3,700$) each were randomly taken from the cortex area of recovering thymi of days 0, 3, 5 and 7. The number and the size of vacuoles in the CTECs were assessed, and the average was calculated for each day. Statistical significance was determined by Student's *t*-test.

Ethical consideration

This experiment was reviewed by the Ethics Committee for Animal Experimentation of the Yamaguchi University School of Medicine, and carried out according to the Guidelines for Animal Experimentation of the Yamaguchi University School of Medicine and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Results

The ultrastructure of cortical thymic epithelial cells of the normal rat

The ultrastructure of individual CTEC subtypes has been described elsewhere in a previous paper (Arudchelvan *et al.*, 2002). Among them, the most typical CTECs were the intermediate type which was characterized by its irregular shape with long cytoplasmic processes extending in various directions. The cytoplasm contained tonofilaments, desmosomes, and a moderate number of vacuoles, mitochondria, endoplasmic reticulum (ER), and lysosomes (Fig. 1A).

Fig. 1. A: Normal rat thymic cortex. The cortical thymic epithelial cell (CTEC) is characterized by the presence of tonofilaments (large arrowhead), a prominent nucleus (n), and small electron-lucent vacuoles (v). The vacuoles contain electron-dense granular materials (small arrowhead) along their inner aspect of the limiting membrane. Asterisk: mitochondria, arrow: rough endoplasmic reticulum (rER), th: thymocyte. **B:** The CTECs three days after irradiation. Note that the number, size, and electron-dense granular content of cytoplasmic vacuoles (v) are greatly increased. The Golgi apparatus (arrow) is located in close proximity to a vacuole. n: nucleus of CTEC, arrowheads: desmosomes. A and B: $\times 5,000$

Fig. 1. Legend on the opposite page.

Fig. 2. Legend on the opposite page.

Alterations in the cortical thymic epithelium of irradiated and recovering rats

The most commonly observed cells during the third and fifth days of recovery were intermediate subtypes of CTECs (Arudchelvan *et al.*, 2000). Proliferating thymocytes were observed on days 3 (Fig. 3A) and 5 in the vicinity of the CTECs. A number of phagosomes sometimes containing remnants of dead thymocytes were found abundantly within the cytoplasm of cortical macrophages (Fig. 3B). The ultrastructural changes of the thymocytes and these stromal cells have already been documented in our previous paper (Arudchelvan *et al.*, 2000). The morphological alterations in the CTECs were most prominent during the third to fifth day of recovery. In particular, the CTECs displayed the characteristics of hypertrophic changes, i.e., the appearance of an increased number of well-developed vacuoles (Fig. 1B).

More precisely, the increased vacuolization was first noticed as early as the third day after irradiation and reached the highest degree on the fifth day, but the extent of vacuolization gradually decreased thereafter. Semiquantitative analysis showed that the number of vacuoles increased significantly on days 3 and 5 (Table 1).

Table 1. Semiquantitative assessment of vacuoles in cortical thymic epithelial cells.

Days after Irradiation	Number of vacuoles	Size of vacuoles (mm)
0	6 ± 1.0	8.9 ± 1.7
3	16 ± 3.6*	11.6 ± 0.9
5	10 ± 1.0*	11.3 ± 2.7
7	7 ± 1.0	9.3 ± 2.5

Values are mean ± SD (n=3).

*P<0.01; level of significance relative to day 0 (control).

Similarly, there was an increase in the size of the vacuoles on days 3 and 5 but the differences were not significant as compared with normal rats (day 0). The vacuolization of the CTECs included the increase not only in the number and size of vacuoles, but also in the amount of their content (Fig. 1B). Some of these vacuoles were fused with one another to form larger vacuoles (Fig. 2C) — and sometimes giant vacuoles (Fig. 2A). The granular content of the vacuoles appeared not only as a loosely aggregated form or finely dispersed granules but also as dense bodies (Fig. 2C). In contrast to the usually empty vacuoles, those of the irradiated group contained vesicular (Fig. 2B) and irregular membranous structures (Fig. 2C) or myelin figures (structures that have a similar morphology to the myelin sheath around peripheral nerve axons) (Fig. 2D). On the seventh day of recovery the ultrastructural appearance of CTECs returned almost to normal. There were no noticeable changes in the mitochondria, ER, lysosomes, or other organelles.

Discussion

The salient features of the morphological alterations in the CTECs during the recovery were the increase in number and size of the vacuoles, and the increase in amount of their content, especially of the granular content. Although not much is known about the nature and function of vacuoles, it is considered that at least some of them may be storage vacuoles of secretory products although they do not have the ordinary morphology of secretory granules (Savino and Dardenne, 1986; Nabarra and Andrianarison, 1987). Strong evidence has been accumulated that the vacuole in the normal TECs represents the site where thymic hormones like thymulin (FTS) are stored and where they mature (Auger *et al.*, 1982; Savino and Dardenne, 1986; Nabarra and Andrianarison, 1987). Furthermore, our recent immunoelectron microscopic study shows that certain vacuoles also act as conventional endocytic compartments, and they may represent the MHC class II compartment of

Fig. 2. A: A CTEC three days after irradiation. Note the giant vacuole (v) within the cytoplasm. Arrowhead: tonofilaments, n: nucleus of the CTEC. **B:** A CTEC five days after irradiation. Vesicular structures (arrowhead) can be seen within vacuoles of the CTEC. **C:** A CTEC three days after irradiation. The content of vacuoles in the CTEC takes various forms. The most common type of content is observed as fine granular materials (asterisk); membranous structures (large arrowheads) and dense bodies (small arrowhead) are also found within some vacuoles. Some vacuoles are in the process of fusing with each other (arrow). A large vacuole (asterisk) appears to be formed by the fusion of vacuoles. Some vacuoles (v) are located in indentations of the nucleus, n: nucleus of CTEC, ncl: nucleolus. **D:** A CTEC three days after irradiation. Myelinoid structures are also occasionally seen in the vacuole (arrowhead). The arrow indicates a vacuole containing a dense body, vesicles, and membranous structures. A–D: ×5,000

Fig. 3. The rat thymic cortex five days after irradiation. **A:** A dividing thymocyte (asterisk) can be seen in the vicinity of the cell process (small arrows) of a CTEC. Arrow: desmosome. **B:** A macrophage (m) contains debris of phagocytosed cells which are presumed to be thymocytes. p: phagosomes. $\times 5,000$

CTECs (Arudchelvan *et al.*, 2003).

Evidence obtained in the present study suggests that the morphological alterations of CTECs after irradiation may occur due to increased cellular activities. The CTECs, after irradiation, were characterized by an increase in the size of

their cytoplasm and the presence of well-developed vacuoles. The features of the CTECs in the present study resemble the hypertrophic TECs of a study of splenectomized chickens (Rusu *et al.*, 1981) and cyclophosphamide-treated rats (Yoon *et al.*, 1997) showing various signs of secretory

activity with a cytoplasm containing numerous large vacuoles. As suggested by these authors, we consider that the hypertrophic epithelial cells have an intense metabolic activity and that they synthesize protein. Interestingly, a series of our previous studies on the irradiated (6Gy) thymus showed that the expression of hormones like FTS (Wang *et al.*, 1999) and messages of cytokines like IL-1b, IL-6, TNF (Mizutani *et al.*, 2002; Adachi *et al.*, 2005) were higher on days 3 and 5 during the recovery compared with non-irradiated rats. Furthermore, the MHC class II expression was also higher in the cortex on day 3 compared with non-irradiated rats (Tokuda *et al.*, 2002). Therefore, these facts might also suggest that, during the recovery of thymic cortical thymocytes after destruction by irradiation, the CTECs may have a considerable influence on the process of proliferation, maturation, and differentiation of the thymocyte precursors.

Pinkel (1968) reported that many of the TECs showed evidence of phagocytic activity, in that what appeared to be the parts of lymphocyte nuclei were present within their cytoplasm. However, other investigators have suggested that the TECs are incapable of phagocytic action (Clark, 1963). The present study did not obtain any conclusive evidence of phagocytosis, such as the engulfment of thymocytes in the CTECs. On the other hand, in our preliminary immunoelectron microscopic study, we found that the CTECs are positive for ED1 and ED2, the markers for rat thymic macrophages (Arudchelvan *et al.*, unpublished observations). Further studies are needed to investigate this problem.

Myelin figures are structures similar to lamellar bodies found in the phagocytosing cells. Although the reason for their formation is not well understood, they appear to be cell debris or the remnants of degraded organelles. Because the autophagic vacuoles also often contain similar structures, some vacuoles with myelin figures may be also concerned with autophagy of the CTECs in a hypertrophic state.

In the present study we have mainly focused on the ultrastructural alterations in the cortical thymic epithelial cells. Studies on the alterations in the medullary epithelial cells, macrophages, and dendritic cells will provide further information on events after irradiation.

In conclusion, the present study showed that the morphological features of the CTECs after irradiation were explained by the hyperfunctional activities of the CTECs. The CTECs after irradiation may play a role in the proliferation and maturation of thymocytes — probably mainly through the secretion of humoral factors like hormones and cytokines, by providing MHC molecules, and by other unknown mechanisms. Further studies should provide more insights into these facts.

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