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Originals

Interleukins modulate glucocorticoid-induced thymocyte apoptosis

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Summary. Glucocorticoid hormones, calcium ionophores and anti-CD3 monoclonal antibodies induce apoptosis in mouse thymocytes. This type of cell death, which is characterized by an extensive DNA fragmentation into oligonucleosomal subunits, occurs in the intrathymic process of negative selection, and is involved in the deletion of autoreactive T-cells during thymic maturation. A number of cytokines are able to modulate apoptosis, and interleukins, including interleukin-1, interleukin-2, and interleukin-4, play a crucial role in thymic maturation and T-cell development. We tested the effects of several cytokines on the glucocorticoid hormone-induced apoptosis of mouse thymocytes in vitro, and demonstrated that interleukin-1α, interleukin-2, and interleukin-4 inhibit the apoptosis induced by dexamethasone, but that interleukin-3 and interleukin-6 exert no noteworthy effect. Dose-response experiments indicated that interleukin-4 is more potent than interleukin-1α and interleukin-2 in inhibiting dexamethasone-induced apoptosis. Furthermore, interleukin-4 fully inhibited the DNA fragmentation induced by the protein kinase-C activator 12-O-tetradecanoylphorbol-13-acetate, but was ineffective against apoptosis induced by the calcium ionophore A23187. These results suggest that interleukins regulate the thymic selection process by acting as modulators of the negative selection process.

Key words: Glucocorticoids – Apoptosis – Cytokines – Interleukins – Thymus

Introduction

Apoptosis is a cell death mechanism characterized by chromatin condensation, micronucleation, and fragmentation of DNA into oligonucleosomal subunits. This process is operative during embryogenesis, in tumor regression, and in the elimination of self-reactive T-lymphocytes in the thymus [21–23]. Furthermore, both antigen receptor stimulation [5, 8, 16, 18] and glucocorticoid hormone (GCH) treatment of immature thymocytes [3, 13, 21] lead to internucleosomal DNA cleavage and so to cell death. GCH-induced cell death is an energy-dependent process which requires specific mRNA and protein synthesis, protein kinase-C (PKC)-dependent phosphorylations and activation of Ca^{2+}/Mg^{2+}-dependent endonucleases. Inhibition of mRNA and protein synthesis, PKC inhibitors, and zinc ions counteract dexamethasone (DEX)-induced apoptosis [3, 13, 21]. Activation of the pituitary-adrenal axis results in thymic hypoplasia in mice [15], and physiological concentrations of GCHs are able to induce thymocyte apoptosis. It has been postulated, therefore, that GCHs play a role in the negative selection mechanisms underlying the T-lymphocyte differentiation program [18].

Interleukins (ILs) have been shown to regulate thymocyte differentiation and T-lymphocyte development, and IL-1 and IL-2 protect thymocytes from the apoptosis induced by anti-CD3 monoclonal antibody or GCH [4, 9, 11, 12]. This suggests that the control of apoptotic death is an important mechanism through which ILs regulate the thymic selection process and T-cell maturation [19, 20].

To better evaluate the possible interactions between ILs and GCHs, we studied the effects of different ILs on GCH-induced apoptosis in mouse thymocytes in vitro. Our results show that IL-1α, IL-2 and IL-4 are able to inhibit apoptosis and suggest that these ILs may be involved in the process of thymic selection.

Materials and methods

Cell suspension. Thymocytes were obtained from 2 to 4-week-old C3H/HeN mice purchased from Charles River (Milan, Italy). The animals were killed by cervical dislocation and the thymuses teased in RPMI-1640 medium. The cell suspension was washed, filtered, and adjusted to a concentration of 1.5×10^6 cells/ml in RPMI-1640 medium supplemented with 5% fetal calf serum and 10 mM Hepes buffer.

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Aliquots of 2 ml thymocytes were incubated at 37 °C with DEX concentrations from 10^{-7} to 10^{-11} M, or DEX plus mouse recombinant IL-1α, IL-2, IL-3, IL-4, and IL-6 (Genzyme, Maidstone, UK). At pre-established times, the cells were centrifuged at 200 *g* for 10 min, washed, and processed (see below). In some experiments cell were incubated with different concentrations of the calcium (Ca^{2+}) ionophore calcimycin (A23187) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma, St. Louis, Mo.).

DNA labelling technique and flow cytometry. Apoptosis was measured by cytometry according to a previously published procedure [10]. Briefly, the pellets were gently resuspended in 1.5 ml hypotonic solution of propidium iodide (PI, 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma), and the tubes placed at 4 °C in the dark overnight. The right-angle scatter and PI fluorescence of individual nucleic were measured with a FACSCAN flow cytometer (Becton Dickinson, Mountain View, Calif.). The nucleic traversed the light beam of a 488-nm Argon laser. A 560-nm dichroic mirror (DM 570) and a 600-nm band pass filter (hand width 35 nm) were used for collecting the red fluorescence due to PI staining of DNA, and the data recorded on a logarithmic scale. All data were recorded in a Hewlett Packard (HP 9000, model 310) computer. The percentage of apoptotic cell nuclei (sub-diploid DNA peak in the DNA fluorescence histogram) was calculated using specific research software (FACSCAN and LYSIS II, Becton Dickinson).

Statistical analysis. Results are the mean ± SD. Due to the non-normal distribution of the data, non-parametric tests (Kruskall-Wallis analysis of variance) were adopted for statistical evaluation.

Results

Figure 1 shows the results obtained when mouse thymocytes were incubated with medium alone, medium plus 10^{-7} M DEX, or 10^{-7} M DEX plus ILs at a concentration of 100 units/ml. Thymocytes incubated in medium alone displayed two peaks in the DNA fluorescence histograms; the normal cells gave a high, narrow diploid peak, while that of the hypodiploid apoptotic cells was low and broad. The percentage of nuclei with diploid DNA content was greatly reduced in DEX-treated thymocytes and the hypodiploid peak markedly increased. Apoptosis was reduced by IL-4 and to a lesser extent by IL-1α and IL-2, whereas IL-3 and IL-6 induced no detectable inhibition of nuclear fragmentation.

These results were corroborated by dose-response experiments. Results reported in Fig. 2 show the effects of different IL doses (from 5 to 500 units/ml) on DEX-induced thymocyte apoptosis. A strong antiapoptotic effect of IL-4 was confirmed, and the lowest concentration we used (5 units/ml) was still able to counteract the DEX-induced apoptosis. Only the higher concentrations of IL-1α and IL-2 inhibited apoptosis, while no concentration of IL-3 or IL-6 exerted any effect on DNA fragmentation.

Since IL-4 produced the maximum-anti-apoptotic effect, its action on thymocyte apoptosis induced by various substances was investigated. For this purpose, we evaluated the effect of 100 units/ml IL-4 on DNA fragmentation induced by different doses of DEX, or the PKC activator TPA, or the Ca^{2+} ionophore A23187, which increases the cell Ca^{2+} content and directly activates the endonucleases. Figure 3 shows the results of these experiments. IL-4 afforded significant protection against the apoptosis induced by nanomolar to micromo-

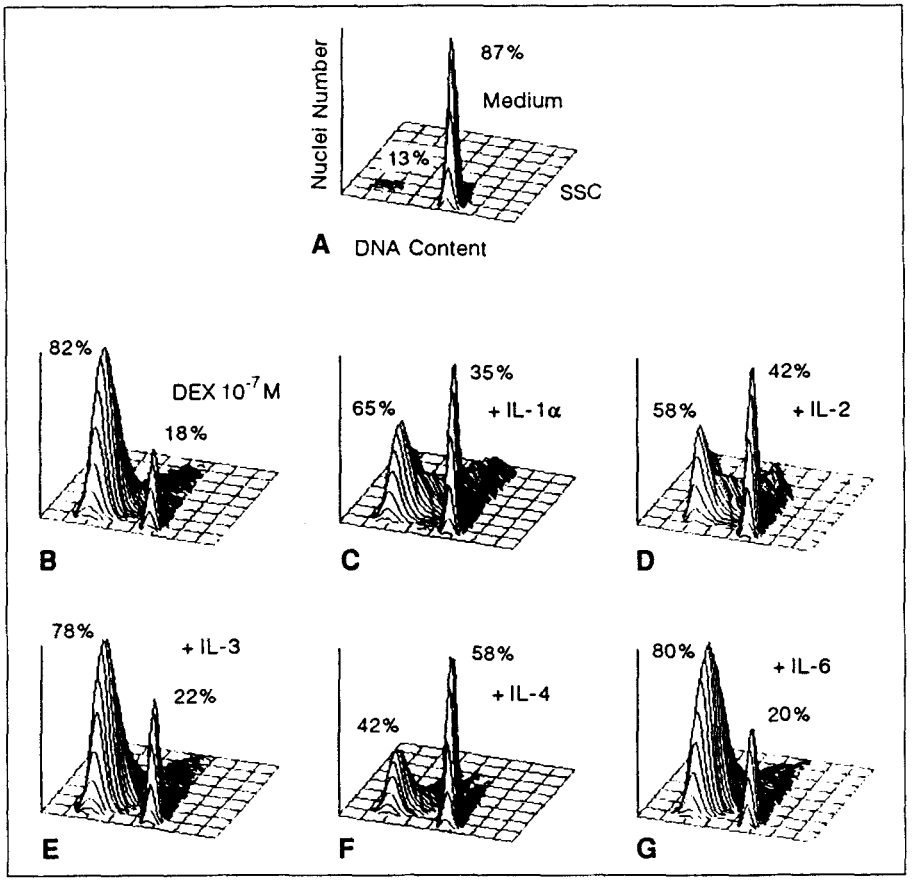

Fig. 1A–G. Computer-drawn three-dimensional flow-cytometric profiles of propidium iodide-stained thymocytes after 24 h incubation in medium alone or indicated substances. The DNA fluorescence of nuclei is plotted against the respective side-angle scatter (*SCC*, chromatin condensation). Apoptotic nuclei have a reduced DNA fluorescence and an increased chromatin condensation. **A** Medium alone; **B** medium plus 10^{-7} dexamethasone (*DEX*) **C** as **B** plus interleukin (IL-1α) (100 units/ml); **D** as **B** plus IL-2 (100 units/ml); **E** as **B** plus IL-3 (100 units/ml); **F** as **B** plus IL-4 (100 units/ml); **G** as **B** plus IL-6 (100 units/ml)

lar concentrations of DEX (Fig. 3A) and entirely blocked the chromatin fragmentation induced by the PKC activator TPA (Fig. 3B), but was completely ineffective against the apoptosis induced by A23187 (Ca^{2+} ionophore) treatment (Fig. 3C).

Discussion

Apoptotic cell death is a physiological process in which cells are selectively deleted from a population in response to specific signals. Apoptosis plays an important role in a wide variety of processes, including embryogenesis, morphogenesis, cell-mediated immunity, tumor regression, normal tissue turnover, and hormone-induced tissue atrophy [22, 23]. This cell death mechanism is involved in the negative selection process that operates in the thymus during T cell ontogeny [19, 20, 24]. Stimulation of the CD3/T-cell receptor (TCR) complex of T-cell precursors by anti-CD3 monoclonal antibodies [8, 18] and exposure of thymocytes to antigen [5] induce DNA fragmentation and cell death. GCHs induce apoptosis in immature thymocytes both in vitro and in vivo, even at physiological GCH concentrations [3]. This suggests that they are implicated in the regulation of thymocyte selection and T-cell development. The antigen-TCR interaction can influence thymic cell development both positively (clonal expansion) and negatively (clonal deletion), depending on the affinity of the antigen-TCR complex and the effect of cooperative signals [25]. GCH could either enhance the antigen-induced negative selection and/or counteract the antigen-induced positive selection process.

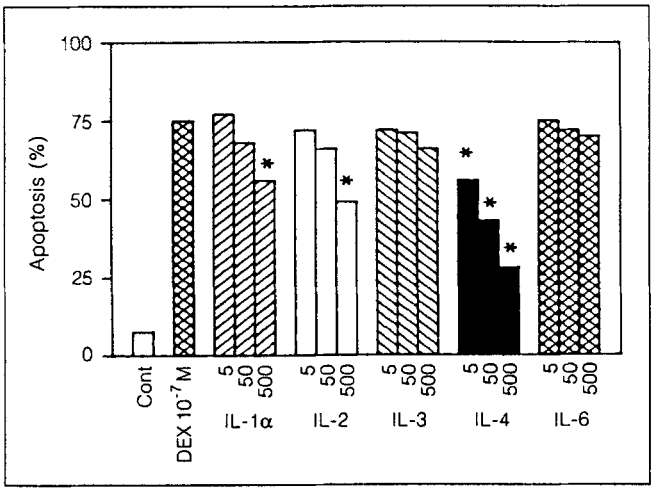

Fig. 2. Dose-response effect of various cytokines on DEX-induced thymocyte apoptosis. Thymocytes were incubated for 24 h in medium alone, medium plus DEX 10^{-7}, or medium plus DEX and different concentrations of ILs. The *bars* are the mean of a representative triplicate experiment. Apoptosis was measured by flow cytometry [10]

ILs are also involved in thymocyte selection and T-cell development. They act as regulators of thymocyte proliferation, and both IL-1α and IL-2 can influence in vitro thymocyte survival by inhibiting apoptotic cell death [4, 9, 11, 12]. It would, therefore, seem that in vivo IL-1α and IL-2 are involved in the signalling pathway of the positive selection process [7]. Our present results demonstrate that IL-1α, IL-2, and IL-4 are able to protect thymocytes against GCH-induced apoptosis, while IL-3 and IL-6 are not. IL-4 exerted a far greater effect than either IL-1α or IL-2, and the effect was still detectable when low (5 units/ml) IL-4 concentrations were used. Although these results were obtained in vitro, they suggest that IL-4 is physiologically implicated in the process of intrathymic

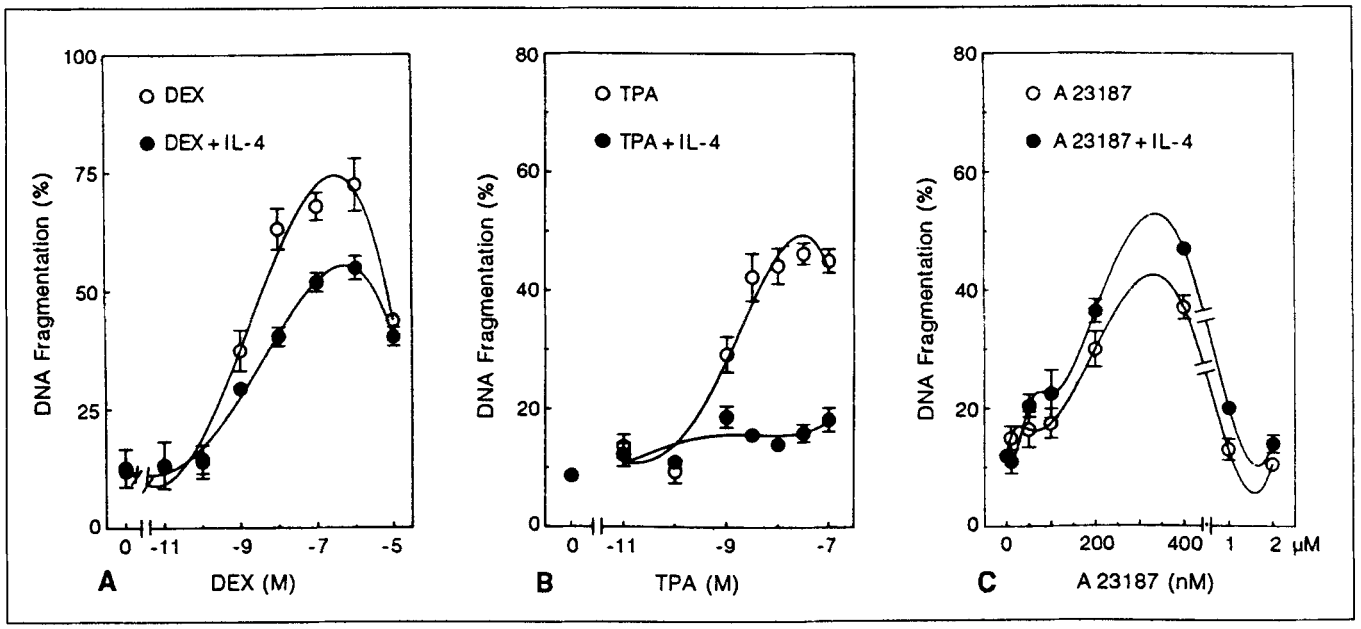

Fig. 3. Effect of IL-4 on DEX-induced (**A**), 12-O-tetradecanoylphorbol-13-acetate (*TPA*)-induced (**B**), or A23187-induced (**C**) apoptosis. Thymocytes were cultured with different concentrations of DEX, TPA, or A23187 inophore with or without IL-4 (100 units/ml) for a 24-h period. The percentage of apoptotic nuclei was evaluated by flow cytometry

selection of T-cell precursors. Support for this concept comes from the demonstration that immature thymocytes express high-affinity IL-4 receptors and IL-4 is an essential growth factor in the early stages of T-cell differentiation [1, 2, 14, 17, 26].

Since PKC activators and Ca^{2+} ionophores have been demonstrated to induce apoptosis in mouse thymocytes in vitro [3, 8, 13], the mechanisms responsible for the anti-apoptotic activity of IL-4 were investigated by testing 100 units/ml IL-4 against increasing doses of DEX, the PKC activator TPA, or the Ca^{2+} ionophore A23187. The results showed that IL-4 significantly inhibited DEX-induced apoptosis and fully abrogated the DNA fragmentation induced by TPA. However, it failed to reduce apoptosis when the Ca^{2+}/Mg^{2+}-dependent endonucleases were directly activated by the Ca^{2+} ionophore A23187. Although the post-receptor events induced by the binding of IL-4 to specific receptors are still largely unknown, our findings indicate that the IL-4-activated pathway(s) interacts physiologically with PKC system(s) to regulate thymocyte apoptosis.

In conclusion, our data provide evidence that IL-1α and IL-2 modulate thymocyte apoptosis. They demonstrate that IL-4 has a strong anti-apoptotic effect and indicate that it exerts its activity by interfering with PKC-dependent pathways. This newly described function of IL-4 is further testimony that this cytokine contributes to controlling the pathway along which a thymocyte must pass to became a fully immunocompetent T-cell.

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References

1. Barcena A, Toribio ML, Pezzi L, Martinez-A C, A role for interleukin 4 in the differentiation of mature T cell receptor gamma/delta + cells from human intrathymic T cell precursors. J Exp Med 172:439, 1990
2. Brown M, Hu-li J, Paul WE, IL-4/B cell stimulating factor 1 stimulates T-cell growth by an IL-2-independent mechanism. J Immunol 131:504, 1988
3. Cohen JJ, Duke RC, Glucocorticoid activation of a calcium dependent endonuclease in thymocyte nucleic leads to cell death. J Immunol 132:38, 1984
4. Fernandez-Ruiz E, Rebollo A, Nieto MA, Sanz E, Somoza C, Ramirez F, Lopez-Rivas A, Silva A, IL-2 protects T cell hybrids from cytolytic effect of glucocorticoids. Synergistic effect of IL-2 and dexamethasone in the induction of high-affinity IL-2 receptors. J Immunol 143:4146, 1989
5. Jenkinson EJ, Kingston R, Smith CA, Williams GT, Owen JJT, Antigen-induced apoptosis in developing T cells: a mechanism for negative selection of the T cell repertoire. Eur J Immunol 20:2517, 1990
6. Kappler JW, Roehm N, Marrak P, T-cell tolerance by clonal elimination in the thymus. Cell 49:273, 1987
7. Lawetzky A, Kubbies M, Hunig T, Rat "first wave" mature thymocytes: cycling lymphoblasts that are sensitive to activation-induced cell death but rescued by interleukin-2. Eur J Immunol 21:2599, 1991
8. McConkey DJ, Hartzell P, Nicotera S, Orrenius S, Jondal M, Calcium-dependent killing of immature thymocytes by stimulation via CD3/T cell receptor complex. J Immunol 143:1801, 1989
9. McConkey DJ, Hartzell P, Chow SC, Orrenius S, Jondal M, Interleukin 1 inhibits T cell receptor-mediated apoptosis in immature thymocytes. J Biol Chem 265:3009, 1990
10. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C, A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271, 1991
11. Nieto MA, Lopez-Rivas A, IL-2 protects T lymphocytes from glucocorticoid-induced DNA fragmentation and cell death. J Immunol 143:4166, 1989
12. Nieto M, Gonzales A, Lopez-Rivas A, Diaz-Espada F, Gambon F, IL-2 protects against anti-CD3-induced cell death in human medullary thymocytes. J Immunol 145:1364, 1990
13. Ojeda F, Guarda MI, Maldonado C, Folch U, Protein kinase-C involvement in thymocyte apoptosis induced by hydrocortisone. Cell Immunol 125:535, 1990
14. Palacios R, Sideras P, Von Boehmer H, Recombinant interleukin-4/BSF-1 promotes growth and differentiation of intrathymic T cell precursors from fetal mice in vitro. EMBO J 6:91, 1987
15. Sei Y, Yoshimoto K, McIntyre T, Skolnick P, Arora PK, Morphine-induced thymic hypoplasia is glucocorticoid-dependent. J Immunol 146:194, 1991
16. Shi Y, Sahai BM, Green DR, Cyclosporin A inhibits activation-induced cell death in T cell hybridomas and thymocytes. Nature 339:625, 1989
17. Sideras P, Funa K, Zalcberg-Quintana I, Xanthopoulos KG, Kisielow P, Palacios R, Analysis by in situ hybridization of cell expressing mRNA for interleukin 4 in the developing thymus and in the peripheral lymphocytes from mice. Proc Natl Acad Sci USA 85:218, 1988
18. Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JJT, Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature 337:181, 1989
19. Sprent J, Lo D, Gao E, Ron Y, T cell selection in the thymus. Immunol Rev 101:173, 1988
20. Von Boehmer H, Teh HS, Kisielow P, The thymus selects the useful, neglects the useless and destroys the harmful. Immunol Today 10:57, 1989
21. Willie AH, Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555, 1980
22. Willie AH, Kerr JFR, Currie AR, Cell death: the significance of apoptosis. Int Rev Cytol 68:251, 1980
23. Willie AH, Morris GR, Smith AL, Dunlop D, Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:67, 1984
24. Zinkernagel RM, Thymuses nd lymphohemopoietic cells. Maturation in selection of T cells, H-2 restriction specificity and H-2 linked Ir gene control. Immunol Rev 42:224, 1978
25. Zircher H, Rohrer UH, Moskophidis D, Zinkernagel RM, Hengartner H, Lower receptor avidity required for thymic clonal deletion than for effector T-cell function. Nature 351:1482, 1991
26. Zlotnik A, Ramson J, Frank G, Fischer M, Howard M, IL-4 is a growth factor for activated thymocytes: possible role in T-cell ontogeny. Proc Natl Acad Sci USA 84:3856, 1987