Cell Death and Differentiation (1999) 6, 593–595

http://www.stockton-press.co.uk/cdd

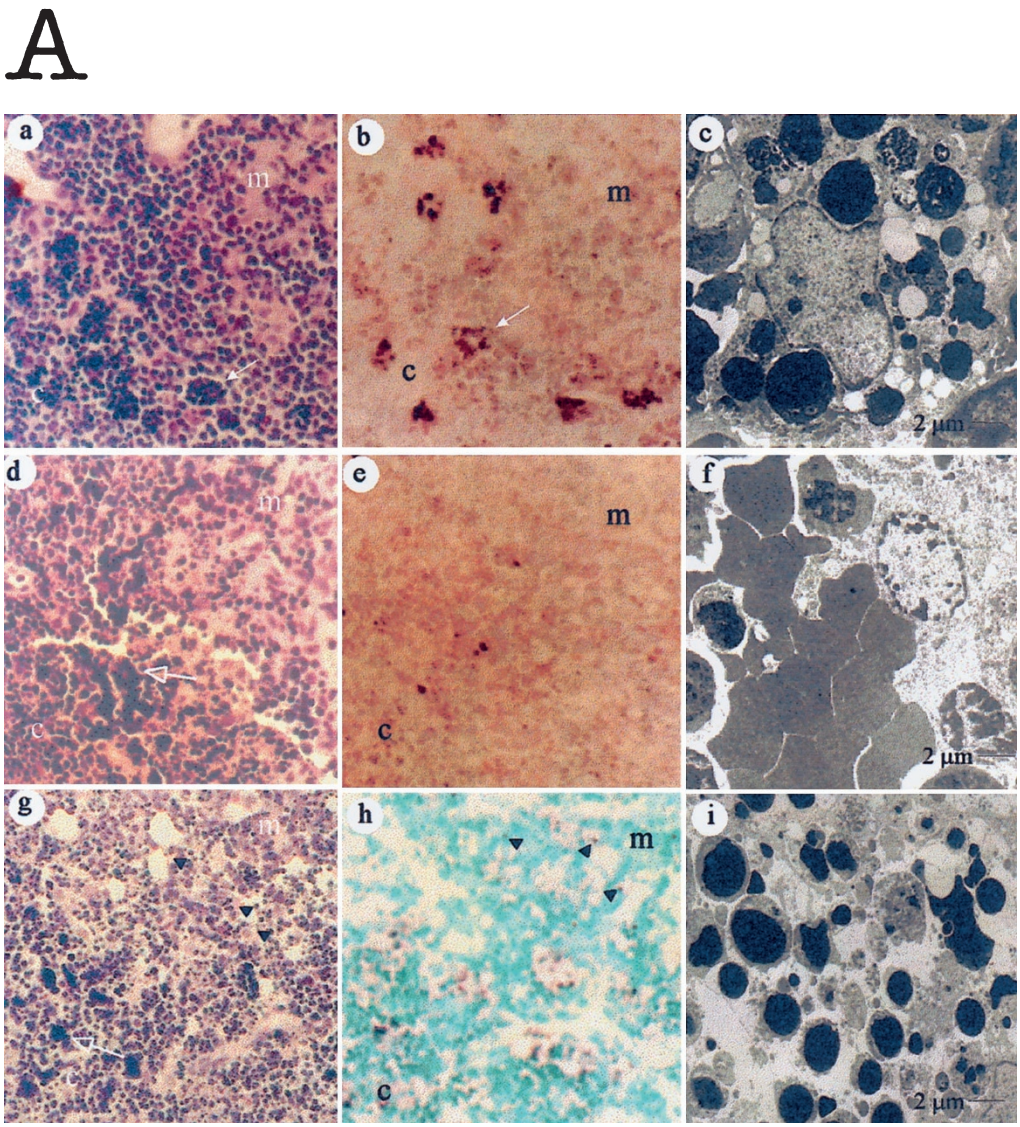
Letter to the Editor

Clearance of apoptotic thymocytes is decreased by inhibitors of eicosanoid synthesis

Dear Editor,

Apoptosis is rarely seen in living tissues. The dead cells are rapidly phagocytosed utilizing biochemical processes on the surface of both dying and engulfing cells.[1–3] Regulation of these processes is poorly understood, blockade of phagocytosis in mammals have not been achieved so far.[4] Here we report that the level of arachidonic acid is elevated in involuting mouse thymus and inhibitors of the formation of eicosanoids can block phagocytosis of dead thymocytes.

Injection of dexamethasone (Dex) into 3–4-week-old mice leads to thymus involution as a result of extensive apoptosis of cortical thymocytes (Figure 1A: a–c and B).[5] They are deleted as clusters of corpses in massively distended phagocytes.[6] We have measured non-esterified arachidonic acid by HPLC[7] and found increased concentration in involuting thymus and a tripled amount in their extracellular space (Figure 1C). Though dexamethasone-



B

	Treatment				
	Dex + solvent	*Dex + AA-861 (60 mg/kg)*	*Dex + Baicalein (70 mg/kg)*	*Dex + Indomethacin (50 mg/kg)*	*Dex + Aspirin (50 mg/kg)*
Involution (%)	67±14	58.4±8.5	49.4±13.7	22.2±17.4	27.2±12.8
Apoptosis					
• FACS	52.1±8.4	51.3±11.1	51.6±5.7	58.2±9.1	54.2±8.7
• DNA degradation	48.9±5.9	51.9± 7.4	43.2±7.6	47.4±5.1	46.7±9.5
Clusters of corpses	58.0±25.1	16.3±6.6	13.3±5.6	23.0±8.0	19.7±8.9
Clumps of non-phagocytosed corpses	4.3±4.1	70.0±9.8	90.0±51.1	46.3±5.5	37.6±12.9

C

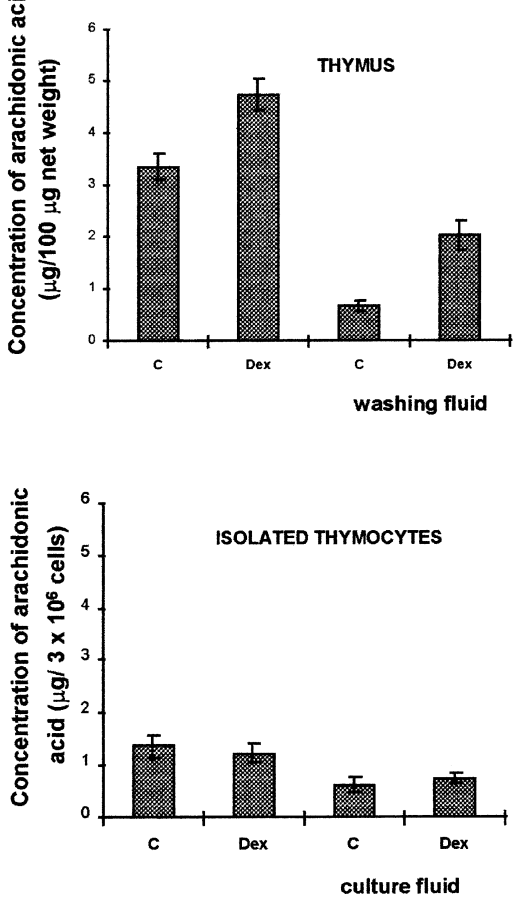


Figure 1 (**A**) Appearance of non-phagocytosed apoptotic cells in Dex-treated mice receiving LOX or COX inhibitors. Hematoxylin eosin staining (a, d and g), TUNEL reaction (b, e and h; the latter was counter-stained by methyl-green) and electron microscopic analysis (c, f and i) of involuting thymus (m: medulla). Mice were treated with dexamethasone (5 mg/kg body weight) and with solvent (a–c), the LOX inhibitor AA-891 (d–f) or the COX inhibitor indomethacin (g–i) 12 h before sacrifice. Clusters of engulfed apoptotic cells (white arrows), clumps of nonphagocytosed dead cells (open white arrows) and individual apoptotic thymocytes outside of macrophages (triangles) are shown. (**B**) Effect of LOX and COX inhibitors on involuting thymus. Involution was assessed as loss of thymic wet weights within 12 h following the administration of dexamethasone as compared to those receiving only solvent. Apoptosis was measured using freshly prepared thymocytes from involuting thymus. Clusters of phagocytosed and clumps of non-phagocytosed apoptotic bodies (defined as at least three corpses attached to each other) were counted on five microscopic fields (magnification 40×) of three sections in each case. Results are shown as mean±S.D. of experiments from at least five mice. (**C**) Concentration of arachidonic acid in involuting thymus and thymocyte cultures. The percentage of apoptotic cells in thymic samples was very close to those shown in (**B**). Primary cultures of thymocytes were maintained for 6 h (**C**) and contained 11–15% dead cells (spontaneous apoptosis), while this was increased to 55–67% in cultures treated with dexamethasone (1 μM). Results are shown as mean±S.D. of five experiments

treated thymocytes undergo rapid apoptosis in *ex vivo* culture no increase of arachidonic acid concentration has been observed in dying cells or their culture media as compared to non-treated controls. Therefore, *in vivo* regulatory roles of eicosanoids formed from the released arachidonic acid have been supposed.

Injection of lipoxygenase (LOX) or cyclooxygenase (COX) inhibitors with dexamethasone has resulted in decreased thymic involution. The 5-lypoxygenase inhibitor AA-861 has influenced involution to a small degree, the 12-lypoxygenase inhibitor balcalaine prevented it more, while the COX inhibitor indomethacin was most effective (Figure 1B). Dex-induced involution was also inhibited by the less specific LOX inhibitor esculetin (data not shown) and the COX inhibitor aspirin. None of these inhibitors induced apoptosis or any morphologic change when administered alone either *in vivo* or *in vitro*. Furthermore, cell death rates measured by either fluorescence-activated cell sorter (FACS)[8] or degradation of DNA[8] were not changed by these treatments either *in vivo* (Figure 1B) or *in vitro* (data not shown) suggesting that the clearance of apoptotic cells was influenced.

Phagocytosis and formation of clusters of engulfed apoptotic bodies were prevented to a significant degree by both LOX and COX inhibitors (Figure 1A: d–i and B). The majority of dead cells was localized outside of phagocytes and the non-phagocytosed apoptotic bodies often formed clumps (Figure 1A: d, f and g) which could be counted quantifying the effect of the inhibitors (Figure 1B). These clumps did not show positive TUNEL reaction in most cases (Figure 1A: e) in agreement with published data that only those apoptotic thymocytes which become $TUNEL^+$ are phagocytosed.[6] In the case of indomethacin the number of non-phagocytosed clumps was less but the dead cells, some $TUNEL^+$, appeared in high numbers both in the cortical layer and even in the medulla of the involuting thymus (Figure 1A: g–i).

The concentration of arachidonic acid was also elevated when thymus involution was initiated by either anti-CD3 antibody to induce death through the T cell receptor or by etoposide to activate the P53 pathway.[5] LOX/COX inhibitors did not influence apoptosis rate after these treatments but inhibited involution to varying degrees and blocked phagocytosis in a fashion similar to that observed using dexamethasone (data not shown).

Our results suggest that eicosanoids may have regulatory roles in the physiologic clearance mechanism of apoptotic cells and inhibition of their formation leads to the inhibition of phagocytosis under *in vivo* conditions – a phenotype similar to that observed in phagocytosis mutants of the nematode *Caenorhabditis elegans*.[1] Future studies should reveal which molecular mechanisms participating in the clearance of apoptotic cells[2–4] are affected by which eicosanoids. In our preliminary study none of the used inhibitors have influenced the externalization of phosphatidylserine on the surface of dying thymocytes (data not shown). It should be noted that LOX and COX enzymes, respectively, might not be the only molecular targets of the compounds used. It has been recently reported that the COX inhibitors aspirin and salicylate inhibit the activity of IκB kinase-β[9] and the complex biochemical pathways of the clearance of apoptotic cells[1–4] may provide targets of the compounds studied here.

The above observations raise the possibility that some of the serious side effects of non-steroidal anti-inflammatory drugs[10] may be related to the inhibition of phagocytosis of apoptotic cells in some tissues. Clearance of dead cells eventually takes place in mouse thymus in the presence of the studied inhibitors of eicosanoid formation; repeated injections or higher dosage of the inhibitors only could slow down but not prevent the involution process. The most likely mechanism of clearance under such conditions is into the blood circulation – through the interstitial fluid ('lymphatics')[11] – as suggested by frequent appearance of apoptotic cells in and close to vessels. Nevertheless, the possible disturbance of the natural clearance mechanism may lead to inflammatory and autoimmune responses[2–4] because of the leakage of noxious contents and the exposure of the dead cell components to the immune system.

Felix Aurel Mic[1]
Peter Molnar[2]
Tamas Keresztes[3]
Eva Szegezdi[1]
Laszlo Fesus[*,1]

[*,1]Department of Biochemistry and Molecular Biology, University Medical School, Debrecen, Hungary H-4012,
[2]Department of Pathology, University Medical School, Debrecen, Hungary H-4012,
[3]Central Service Laboratories, University Medical School, Debrecen, Hungary H-4012
*Corresponding author
Permanent address of FA Mic: Institute of Public Health, Timisoara, Romania 1900

1. Ellis RE, Juan J and Horvitz HR (1991) Annu. Rev. Cell Biol. 7: 663–698
2. Savill J and Haslett C (1995) Semin. Cell Biol. 6: 385–393
3. Platt N, da Silva RP and Gordon S (1998) Trends Cell Biol. 8: 365–371
4. Gregory CD (1998) Cell Death Differ. 5: 549–550
5. Szondy Z, Molnar P, Nemes Z, Boyiadzis M, Kedei M, Toth R and Fesus L (1997) FEBS Letts. 404: 307–313
6. Nakamura M, Yagi H, Ishii T, Kayaba S, Soga H, Gotoh T, Ohtsu S, Ogata M and Itoh T (1998) Eur. J. Immunol. 27: 999–1004
7. Wang Y, Kay HH and Killam AP (1991) Am. J. Obstet. Gynecol. 164: 812–818
8. Szondy Z, Reihert U, Bernardon JM, Michel S, Toth R, Ancian P, Ajzner E and Fesus L (1997) Mol. Pharmacol. 51: 972–982
9. Yin MJ, Yamamoto Y and Gaynor RB (1998) Nature 396: 77–80
10. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van de Putte LBA and Lipsky P (1998) FASEB J. 12: 1063–1073
11. Suster S and Rosai J (1997) In: Histology for Pathologists. (ed Sternberg, SS). pp 687–706. Lippincott-Raven, Philadelphia-New York.