

Short communication

Flow cytometric analysis of spontaneous and dexamethasone-induced apoptosis in thymocytes from severely malnourished rats

Rocío Ortiz*, Leticia Cortés, Humberto González-Márquez, José Luis Gómez, Cristina González and Edith Cortés

Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Apdo. Postal 55-535, C.P. 09340, México D.F., México

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Severe malnutrition is widely distributed throughout the world, showing a high prevalence in developing countries. Experimental animal models have been useful to study the effects of malnutrition at different levels and ages. Apoptosis is a well recognised process of cell death occurring under several physiological and pathological conditions. It represents the principal mechanism involved in cell selection in the thymus. Thymocyte apoptosis induction by dexamethasone is one of the best characterised experimental models of programmed cell death. The aim of the present study was to determine whether severe malnutrition increased spontaneous and/or dexamethasone-induced apoptosis *in vivo* in thymocytes of experimentally malnourished rats during lactation. Thymocytes were obtained from malnourished rats at weaning (21 d of age). Apoptosis frequency was estimated by the terminal transferase-mediated dUTP nick end labelling assay. Spontaneous apoptosis was 1.9 (SD 1.0) % in well nourished rats in contrast to 13.3 (SD 3.8) % in malnourished animals; this is seven times greater ($P < 0.001$). Interestingly, the frequency of dexamethasone-induced apoptosis was similar in both groups of animals (47.9 (SD 10.1) % in well nourished rats and 53.8 (SD 8.0) % in malnourished rats). The results obtained in the present study indicate that malnutrition is associated with a significant increase of spontaneously apoptotic cells. In addition, the data showed that the fraction of thymocytes susceptible to dexamethasone-induced apoptosis was similar in well nourished and malnourished animals. The greater levels of spontaneously apoptotic cells associated with malnutrition could be related to alterations of the microenvironment of the thymus and/or to an obstruction of early thymocyte maturation.

Apoptosis: Rat thymocytes: Experimental malnutrition: TUNEL assay

Severe malnutrition occurs as a consequence of deficient food intake and/or low-protein diets. Malnutrition is widely distributed throughout the world and has a high prevalence in developing countries. Furthermore, the frequency of this condition is rapidly increasing in these countries, mainly owing to poverty, unemployment and ignorance (De Mello, 1994). The effects of malnutrition may be particularly devastating during childhood, when growth is faster, development of different tissues is active and nutrient requirements are greater (Cravioto & Arrieta, 1985).

Laboratory animals have been useful in studying the

effects *in vivo* and *in vitro* of malnutrition at different levels, since extranutritional factors affecting man may be controlled (Galler & Kanis, 1987; Ortiz *et al.* 1996). Several studies have shown that lactation is critical for the processes of growth and development. The effects of malnutrition are more severe during this period than those observed in adults (Winick & Noble, 1966; Fló *et al.* 1991).

Lymphoid atrophy is a well recognised consequence of nutritional deprivation in animals, including man (Chevalier, 1997). This loss of lymphoid tissue associated

* Corresponding author: Dr Rocío Ortiz, fax +52 58 04 47 27, email arom@xanum.uam.mx

with malnutrition is particularly pronounced in the thymus. This organ is crucial to T-cell development; it provides the specialised microenvironment required for T-cell maturation and the generation of a diverse T-cell receptor repertoire (Anderson *et al.* 1998).

Apoptosis, a well recognised process of programmed cell death, must be regulated both positively and negatively in response to a variety of stimuli in the body. Thymocyte apoptosis represents the main mechanism involved in intrathymic cell selection. It can be induced by a variety of stimuli such as glucocorticoids, ionising radiation, antibodies and toxins, and it is one of the best characterised experimental models of apoptosis.

In the present study, dexamethasone was selected as the apoptosis inducer because of its ability to induce a series of profound biochemical changes in immature thymocytes. However, the pathways beyond receptor transactivation that lead to this form of cell death are not fully understood (Thompson, 1999; Mann & Cidlowski, 2001). Nevertheless, one of the best described characteristics of apoptosis is the selective degradation in the internucleosomal DNA linker regions. Consequently, it can be detected by the TdT-mediated dUTP-X nick end labelling (TUNEL) assay (Sgonc & Gruber, 1998).

The aim of the present study was to determine whether severe malnutrition increased spontaneous and/or dexamethasone-induced apoptosis in the thymocytes of experimentally malnourished rats during lactation.

Materials and methods

Experimental malnutrition during lactation

Wistar rats from the closed colony of breeding at the Universidad Autónoma Metropolitana-Iztapalapa were used. Rats were kept under 12 h controlled light–darkness cycles, at a temperature of 22–25°C, with 45% relative humidity. The nursing mothers had undergone second delivery, were fed with a balanced diet for rodents (Purina Mills International 5001, Richmond, VA, USA) and filtered water *ad libitum*. They were bred in acrylic boxes with beds (Betachips, Northeastern Products Corp., Warrensburg, NY, USA). The experimental procedures were performed according to the guidelines for the use of experimental animals of the Universidad Autónoma Metropolitana-Iztapalapa, which are in accordance with those approved by the National Institutes of Health (Bethesda, MD, USA).

Experimental malnutrition during lactation was induced by food competition (Ortiz *et al.* 1996). Rats (1 d old) from different litters were randomly assigned to two groups. In the well nourished group, pups were assigned to nursing mothers, each suckling six to eight pups. In the experimental or malnourished group, each nursing mother fed fifteen to sixteen pups. Pups were assigned to another nursing mother 1 d after birth, and the mothers accepted the young without demur. The same proportion of male and female pups per litter was assigned to each group. Pups were weighed every 2 or 3 d, and mean body weight was estimated for each litter from the first day until weaning (day 21). Rats were considered as severely malnourished when body weight deficiency was greater than 40% compared to age-matched controls.

Four groups of twelve rats each were studied. Group I contained well nourished rats without treatment; group II contained well nourished rats treated for 20 h with dexamethasone; group III contained malnourished rats without treatment; group IV contained malnourished rats treated for 20 h with dexamethasone. Well nourished and malnourished rats were randomly selected from twelve different litters. Four animals, one from each group, were processed simultaneously.

Cell suspension and treatment

Treated rats were injected intraperitoneally with water-soluble dexamethasone (Sigma Chemical Co., St Louis, MO, USA), 25 mg/kg body weight, 20 h before they were killed by an ether overdose. Control animals (without treatment) received only buffered saline solution.

Thymus cells were obtained by sieving the tissue through a nylon screen (Ortiz *et al.* 1995), and cells were suspended in PBS solution (Ca²⁺- and Mg²⁺-free PBS solution, Microlab, México).

Terminal transferase-mediated dUTP nick end labelling assay

The TUNEL assay was performed as described by Gold *et al.* (1993). The *In Situ* Cell Death Kit (Boehringer Mannheim Biochemica, Germany) was used. Briefly, cell suspensions were placed on ice, fixed, permeabilised, washed and incubated at 37°C for 60 min with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP. The label incorporated at the DNA break sites was visualised by flow cytometry.

Flow cytometry analysis

Flow cytometry analysis was performed with a FACScan flow cytometer (Becton Dickinson, Immunocytometry Systems (BDIS), San Jose, CA, USA) equipped with an argon laser (488 nm). List mode data of 10 000 events were collected for each sample. Analysis was performed using CELL Quest software (BDIS). The marker for determining positive and negative cells was set according to the negative control. The negative region included at least 99% of cells in all cases.

Statistical analysis

Mean, standard deviation and median were calculated according to nutritional status and treatment. Data were analysed to determine statistical significance between groups by Student's *t* test ($P < 0.05$).

Results

Table 1 shows the mean and standard deviation of body weight, thymus weight and number of thymocytes per thymus for all groups of rats. The average body weight of rats 1 d after birth was 7.6 (SD 0.8) g in both groups. At weaning, the average body weight was 20.7 g in

Table 1. Body weight, thymic weight and number of thymocytes per thymus of well-nourished and malnourished rats. Experimental animals were treated with dexamethasone, and control animals were not (Mean values and standard deviations of twelve rats in each group)

	Non-treated				Dexamethasone-treated			
	Well nourished		Malnourished		Well nourished		Malnourished	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Body weight (g)	47.1	5.4	20.5	2.4***	46.5	4.1	20.8	2.6***
Body weight deficit (%)	—	—	56.4	—	—	—	55.3	—
Thymus weight (mg)	105.1	5.3	37.9	12.0***	106.5	19.8	39.4	11.4*
Thymus weight deficit (%)	—	—	63.9	—	—	—	63.0	—
Cells/thymus ($\times 10^6$)	152.85	16.3	53.4	27.5***	104.4	40.7	45.1	5.4

Significant difference between non-treated well nourished v. malnourished: * $P < 0.001$. Significant difference between dexamethasone-treated well nourished v. malnourished: ** $P < 0.001$; *** $P < 0.05$. For experimental procedures, see p. 546.

Table 2. Spontaneous and dexamethasone-induced apoptotic thymocytes in well nourished and malnourished rats (Mean values and standard deviations; medians and ranges)

Apoptosis	Well nourished				Malnourished			
	Mean	SD	Median	Range	Mean	SD	Median	Range
Spontaneous*	1.9	1.0	1.7	1.2–5.0	13.3	3.8	14.8	4.8–19.9
Dexamethasone-induced	47.9	10.1	47.7	33.9–63.8	53.8	8.0	52.6	40.6–69.1

* Significant difference between well nourished and malnourished rats ($P < 0.001$). For experimental procedures, see p. 546.

malnourished and 46.8 g in well nourished rats. The malnourished rats weighed 55.9% less than the well nourished animals, implying severe protein–calorie malnutrition. The average thymic weight was 38.5 mg in malnourished and 105.5 mg in well nourished rats. When both groups were compared, a 63.5% deficit was observed. Thymus weight was similar between non-treated and dexamethasone-treated rats. The average number of thymocytes per thymus was 49.3×10^6 in malnourished and 128.6×10^6 in well nourished rats; the deficit was 61.7%. The number of thymocytes was slightly decreased in both well nourished and malnourished dexamethasone-treated rats, but high variability was evident.

Table 2 shows the mean, standard deviation, median and range of percentage of spontaneous and dexamethasone-induced apoptosis of thymocytes in well nourished and malnourished rats. A significant increase of spontaneously apoptotic cells was found in malnourished rats in comparison with well nourished animals; these percentages were 13.3 and 1.9 respectively ($P < 0.001$). As expected, the *in vivo* treatment with dexamethasone for 20 h increased the level of apoptosis in thymocytes in both groups of rats. The dexamethasone-induced apoptotic rates were near 48% in well nourished rats and 54% in malnourished rats. Surprisingly, the percentages of apoptotic thymocytes in dexamethasone-treated animals were similar ($P > 0.05$).

Discussion

Simon detected the thymus as a sensitive barometer of malnutrition as early as 1845 (cited by Prentice, 1999). However, the molecular mechanisms related to thymus involution are not yet well understood (Prentice, 1999). The

data obtained in the present study showed that thymic atrophy was present in malnourished rats; at weaning the weight of the thymus was considerably decreased. This observation agrees with previous reports (Jambon *et al.* 1988; Fraker *et al.* 1995; Malpuech-Brugere *et al.* 1999).

Thymocytes derived from well nourished rats showed lower spontaneous apoptosis than that observed in thymocytes from experimentally malnourished rats during lactation. This was a general phenomenon, since eleven of the twelve well nourished rats showed lower percentages of apoptotic cells than those obtained from malnourished animals. The rate of spontaneous apoptosis in thymocytes was seven times greater in malnourished rats than in well nourished rats, indicating that malnutrition in 21-d-old rats is associated with an increase in the apoptotic cell fraction in the thymus.

An increased susceptibility to dexamethasone-induced apoptosis *in vivo* in thymocytes from malnourished rats was not detected. It seems that the same fraction of thymocytes is susceptible to activation of apoptosis by glucocorticoids and malnutrition at a certain time. When thymocytes were challenged with dexamethasone in malnourished rats, there were no more thymocytes with apoptotic propensity than were detected in well nourished rats.

The experimental malnutrition model showed that competition for maternal milk during lactation increased the apoptosis rate. This is of the utmost importance, since malnourished rats show several symptoms that are similar to the clinical features in malnourished children (Ortiz *et al.* 1996). On the other hand, experimentally produced deficiencies of specific essential nutrients, e.g. Zn, folate, Mg (Huang *et al.* 1999; Lepage *et al.* 1999; Malpuech-Brugere *et al.* 1999) and/or a failure in molecules that show antiapoptotic effect, e.g. leptin, an apoptosis inhibitor

expressed by macrophages (Miyazaki *et al.* 1999) cause similar effects, but they do not mimic the conditions of malnutrition in childhood.

Furthermore, the involution and atrophy of the thymus gland during ageing and acute starvation have been associated with an altered representation of thymocyte subsets and particularly of CD4+ CD8+ double-positive thymocytes (Provinciali *et al.* 1998; Howard *et al.* 1999). In addition, Miyazaki *et al.* (1999) reported different susceptibility in subsets of immature murine thymocytes to apoptosis induced by dexamethasone.

Preliminary studies of thymocyte subsets in experimentally malnourished rats during lactation indicate that malnutrition is associated with a significant increase in the double-negative subset (CD4− CD8−) and with a significant decrease in the double-positive subpopulation (CD4+ CD8+). The rates of single-positive thymocytes CD4+ and CD8+ were similar in malnourished and well nourished rats (data not shown). Nevertheless, the alteration in thymocyte subsets was less evident than the increase in spontaneous apoptosis observed in the present study. Further studies will be necessary to address the relationship between levels and susceptibility of spontaneous and dexamethasone-induced apoptosis in specific thymocyte subpopulations in 21-d-old malnourished rats.

Conclusion

The results obtained in the present study indicate that, in rats, severe malnutrition induced during lactation was associated with increased levels of spontaneous thymocyte apoptosis. The enhanced level of spontaneous thymocyte apoptosis may be related to the marked thymic atrophy found in the malnourished organism. In addition, the data showed that those thymocyte fractions susceptible to dexamethasone-induced apoptosis were similar between malnourished and well nourished rats.

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