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Preliminary study of osteoblasts in peripheral blood in the population of infants and adolescents*

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Summary
The presence of osteoporosis in adult life is conditional on the adequate development and formation of bone during growth in infancy and adolescence and the successive loss which occurs throughout life. Knowledge regarding bone tissue cells and their precursors in stages of growth is scarce, given the difficulties in obtaining samples of this tissue. Recent studies suggest a method of obtaining osteoblast line cells from peripheral blood. The main objective of this work has been to quantify the osteoblast line cells in the peripheral blood of infants and adolescents, as well as noting any possible differences according to the stage of growth.
38 subjects were studied, 16 children (between 4 and 12 years of age) and 12 adolescents (aged between 12 and 18 years). Osteoblast precursor cells in peripheral blood were analysed using the flow cytometry technique. The preliminary results show higher levels of preosteoblastic cells in the youngest age group: 4.17% ± 0.92 vs 2.03% ± 0.48, p= 0.021. There is a negative correlation between the percentage of pre-osteoblastic cells and age r= -0.488 and weight r= -0.530, p< 0.05. In summary, this technique allows us to quantify preosteoblasts in peripheral blood, and we show that they have a higher percentage, the lower the age, during the period of infancy and adolescence.

Key words: *Osteoblasts, Peripheral blood, Infants, Adolescents.*

Introduction

Osteoporosis is a disease of adult life characterised by the presence of non-traumatic fractures, and among the determining factors of this lower bone resistance one of the most important is the lower bone mineral density of the skeleton. Bone mass develops over a lifetime. During the period of growth bone is formed until it reaches a peak in young adulthood, and subsequently, from 35-40 years of age it is physiology which produces a decrease in bone mass. Therefore it is possible to arrive at a significant reduction in bone mineral density due to two fundamental reasons: low levels of bone formation during the growth period, or excessive bone loss in adulthood[1].

Up to the present time, the majority of the research studies have been centred on the adult stage of bone loss, and little is known about its development and formation at an intimate level during the growth stage of infancy and adolescence, given that for this is would be necessary to carry out bone biopsies, which means carrying out a process which is painfully invasive and not often allowed at this stage of life. However, the physiological knowledge of bone metabolism during the period of growth is of great importance, given that on this depends the bone "capital" which an individual ends up acquiring.

Currently we know that the osteoblast and osteoclast precursor cells, in addition to residing in the bone medulla, are capable of being mobilised through the peripheral blood to be directed to zones in which there is active bone remodelling[2]. The problem is that with the techniques which were being used, the number of osteoblast precursors circulating in the blood described to date, has always been very low and difficult to detect[3,4]. Kosha et al.[2] suggested the possibility of using the technique of flow cytometry, with antibodies for proteins specific to bone (osteocalcin and alkaline phosphatase) to better identify the pre-osteoblast cells circulating in the peripheral blood, and in addition, that if these osteoblast cells have a physiological role in the formation of bone, it is expected that their concentration increases in conditions of bone formation[5,6].

Our principal aim has been to quantify the osteoblast line cells in peripheral blood in healthy children and adolescents, as well as understanding possible differences according to stages of growth and gender.

Material and method

Group studied

We studied 38 subjects (whose parents/guardians had previously given their consent, once informed about the study) divided into two groups according to age: group A (4-12 years), 16 children (7 female, 9 male); group B (13-18 years), 12 adolescents (2 female, 10 male). The period of study was during the year 2008 and the subjects came from the follow up clinic for healthy children or from the ophthalmic service of the Virgen Macarena University Hospital. For all subjects, after carrying out a questionnaire on current and previous state of health, the following actions were carried out: determination of height, weight and body mass index (BMI). In addition, a sample of peripheral blood was taken to determine levels of calcium and bone alkaline phosphatase (BAP) and the levels of pre-osteoblast cells circulating in the blood.

None of the subjects selected had diseases related to bone metabolism, or were taking medicine.

Extraction of mononuclear cells

To obtain the mononuclear cells we diluted the total blood with PBS (1:1). The diluted blood was added to a Ficoll-Hypaque solution 1:2 and centrifuged (1,250 xg, 20 mins 4º C). The ring formed at the interphase contains the mononuclear cells, which are collected and washed with PBS. Subsequently we carried out hypo-osmotic stress with distilled and deionised H_2O to lyse the contaminated red globules, and NaCl at 1.8% was added to re-establish the isomolarity, then after washing with PBS the cells were counted with a haemocytometer, determining their viability by exclusion with Tripan Blue.

Flow cytometry

For the cytometric analysis we incubated the cells with the anti-osteocalcin-ficoeritrine antibody and we selected the reading channel corresponding to the light emitted. The positive population will be identified as cells which express specific levels of fluorescent activity compared with the non-specific autofluorescence of the control isotopes. The cells identified will be expressed as a percentage of the "gate" selected initially corresponding to the area of the lymphocytes-monocytes.

Collection and analysis of data

All the experiments were repeated three times, accepting the value of the arithmetical mean of the repeated exercises. The quantitative values were expressed as an average ± SD.

The software package SPSS v17.0 was used for the management of the statistical results. The ANOVA test was used to compare quantitative variables and Pearson's correlation coefficient for correlation between variables.

In all cases, the level of significance was considered to be 5% ($p<0.05$).

Results

We would like to make it clear that we present here preliminary results, given that at present we are continuing to increase the sample size for all the groups. The average age of the group of children was 9.05 ± 3 years, of whom 7 were girls and 9 boys, of comparable ages. In the group of adolescents, the average age was 14.16 ± 1.2 years, with 2 girls and 10 boys. The characteristics of both groups are given in Table 1. We did not observe significant differences in the vales if BMI, calcium or alkaline phosphatase between the two groups studied, or within the same group, between genders.

The osteoblast line cells were quantified according to the percentage of cells positive for osteocalcin circulating in the peripheral blood, using flow cytometry.

The group of children between 4 and 12 had a significantly higher percentage of pre-osteoblast cells (pre-OB) in their peripheral blood (4.17% ± 0.92) than that found in the adolescents over 12 years of age (2.03% ± 0.48 p= 0.021) (Figure 1).

In analysing the data by sex we did not find differences within the same group. Specifically, in group A the levels of pre-OB were slightly higher in males (4.5% ± 0.9) than in the females (3.34 ± 0.9), even though this difference was not statistically significant. And for group B, the quantity of pre-OB cells was also very similar (1.95 ± 1.96 in boys; 1.06% ± 1.03 in girls) (Figure 2).

We have confirmed the fact that there is a negative correlation between the percentage of osteocalcin-positive pre-osteoblast cells circulating in the peripheral blood, and age (r= -0.488 and p= 0.005), as well as between the number and body weight (r= -0.530, p= 0.035).

Table 1. Anthropomorphic characteristics and blood biochemistry parameters related to calcium metabolism of the groups studied. The results are expressed as an average ± standard deviation

Variables	Group A (4-12 years) n=16	Group B (13-16 years) n=12
Age (years)	9.05 ± 3	14.16 ± 1.2
Size (cm)	136.5 ± 20.5	158.5 ± 9.1
Weight (Kg)	39.26 ± 18	53.5 ± 10.7
BMI (Kg/m^2)	19.69 ± 4.5	21.1 ± 2.55
BOA (U/L)	550 ± 181	576 ± 79.7
Calcium (mg/dl)	9.7 ± 0.38	9.8 ± 0.37

BMI: body mass index
BOA: bone alkaline phosphatase

Discussion

In this work we show that, using flow cytometry techniques, it is possible to quantify osteoblast line cells in the population of young people. In adults it had been indicated that the quantity of these cells was practically undetectable, while increases were seen in patients with bone fractures[3,4]. We consider that the availability of this powerful technique enables us to evaluate in children and adolescents highly important factors related to bone metabolism, an evaluation which would be difficult to carry out in any other way, due to the need to analyse samples obtained through bone biopsies.

We have observed that the number of pre-osteoblasts in peripheral blood is dependent on age, reaching higher values in younger children, ≤ 12 years of age, compared with adolescents of between 12 and 16 years of age. To us, these results could indicate that the more the pre-osteoblast cells in peripheral blood increase the more active is the process of bone remodelling. Children in the most rapid phase of growth have a correspondingly high level of bone remodelling activity, and therefore, a higher number of osteoblast line cells in circulation. Due to this same mechanism, in patients with bone fractures, in whom there is a higher level of bone formation in the area of the skeleton in which the bone callus is formed, has been found a higher percentage of pre-osteoblasts in peripheral circulation, compared with values found in healthy subjects[5].

The number of pre-osteoblast cells in peripheral blood does not only correlate with age, but we have also observed a negative correlation with weight (r= -0.530 p= 0.035). These results may concur because both osteoblasts and adipocytes derive from the same pluripotent mother cells and, as such, a higher differentiation in one direction may be accompanied by a lower proportion of the other cells.

In the developmental stages of an individual, in which the activity of bone formation is higher than the formation of adipose tissue, as is usual in growth during infancy, it is predominantly the cellular signals for the formation and maturation of osteoblasts (an increase in the canonical Wnt signalling pathway with over-expression of Runx2) which need to be activated at the expense of the genesis of adipocytes (reduction in PPARγ2). These data need to be confirmed, but they would relate to a higher level of formation of adipocytes in the bone medulla at the expense of a lower number of osteoblasts, which has been confirmed in the adult population[7].

We found no differences in the quantity of pre-osteoblasts in peripheral blood by gender. During infancy it is known that boys and girls increase bone mass as they grow in height in a similar way. It is from adolescence, under the influence of sexual hormones, when the curves for the increase in BMD are seen to separate, with males achieving higher values[8]. In our study few subjects at this stage were evaluated, and those were mainly female. It is necessary to study a larger population of adolescent girls to reach conclusions about this.

Figure 1. Average value of osteocalcin positive cells for group A (4-12 years) and for group B (13-18 years)

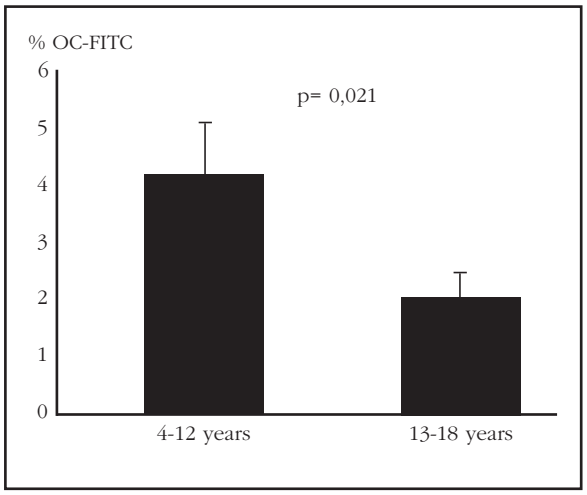
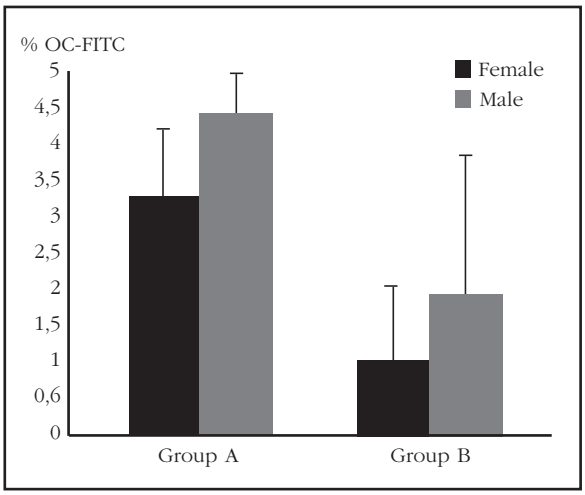


Figure 2. Average value for groups A (4-12 years) and B (13-18 years) separated by sex



We can definitively conclude, despite the small sample size, that by utilising the flow cytometry technique and using osteoblast cell markers, such as osteocalcin, it is possible to quantify a valid percentage of osteoblast line cells in peripheral blood in healthy children and adolescents. In addition, we show that there is a higher percentage of these cells in smaller children, in whom bone formation is more intense, and in inverse relation to body weight.

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