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Mechanisms of enhanced osteoclastogenesis in girls and young women with Turner's Syndrome

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ABSTRACT

Subjects with hypergonadotropic hypogonadism due to Turner's syndrome show low cortical mineral density, osteoporosis and risk of fractures. It is not clear if this bone fragility derives from chromosomal abnormalities or is the result of inadequate bone formation due to estrogen deficiency.

The aim of this study was to investigate the cellular mechanisms underlying bone fragility in subjects with Turner's syndrome before induction of puberty and after hormonal replacement therapy (HRT). For this purpose, we have evaluated the osteoclastogenic potential of non-fractioned and T-cell depleted cultures of peripheral blood mononuclear cells (PBMCs) belonging to girls with Turner's syndrome who had not been treated with HRT yet, girls and young women who were on HRT and age-matched controls. Untreated subjects showed high FSH serum levels, whereas the other subjects displayed normal FSH serum levels. T-cell immunophenotype was analyzed through flow cytometry. Biochemical and DXA analyses were performed.

Spontaneous osteoclastogenesis in non-fractioned and T-cell depleted cultures of PBMC belonging to girls with high FSH levels was more evident than in cultures of subjects with normal FSH levels. In the former, osteoclastogenesis was sustained by monocytes expressing high levels of c-fms, TNF-α and RANK, and T-cells producing high RANKL and TNF-α; in the latter it was supported by T-cells expressing high RANKL levels. $CD4^{+}CD25^{high}$ T-cells were reduced in all subjects, whereas $CD3^{+}/CD16^{+}/CD56^{+}$ NKT-cells were increased in those with high FSH levels. High RANKL and CTX levels were detected in the sera. Bone impairment was already detectable by DXA in subjects aged under 10, although it became more evident with aging.

In conclusion, our results demonstrated that bone fragility in subjects with Turner's syndrome is associated to enhanced osteoclastogenesis. This process seems to be due to high FSH serum levels before HRT, whereas it is caused by high RANKL during HRT.

1. Introduction

Turner's syndrome is a sex-chromosomal disorder resulting from partial or complete X-chromosome monosomy, which occurs in 1:2500 female live births. It is characterized by growth failure, ovarian dysgenesis, cardiac anomalies and neurocognitive problems [1]. Low bone mineral density (BMD) and osteoporosis are clinical features in women with ovarian failure caused by Turner's syndrome, affecting up to 45% of subjects often two to three decades earlier than that noted in postmenopausal osteoporosis [2,3]. The increased bone fragility in Turner's syndrome consists of reduced cortical BMD with normal trabecular bone density [4]. Moreover, these subjects are at risk of

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fractures, which is higher during childhood and after 45 years [5]. Indeed, small bones may be per sé an important risk factor for increased susceptibility to fractures [6]. However, it is not clear whether the low cortical mineral density in subjects with Turner's syndrome is intrinsic to the X-chromosomal abnormality or is the result of delayed and inadequate bone formation which is associated to estrogen deficiency during development and in adulthood [3,7,8]. Although many girls with Turner's syndrome undergo growth hormone (GH) therapy to treat short stature in childhood and estrogen therapy to treat hypogonadism at puberty, the efficacy of these treatments on BMD is controversial [9–12].

Girls with Turner's syndrome have a biphasic pattern of gonadotrophin levels dependent on age and karyotype, with high FSH and LH serum levels during infancy and at the time of expected puberty in X-monosomic subjects respect to those with mosaicism [13]. FSH serum levels, rather than serum estrogens, best correlate with bone turnover markers in pre- and perimenopausal women [14]. Furthermore, hypergonadotropic amenorrheic women show low BMD due to a potential direct effect of FSH on bone metabolism [15]. On the other hand, decreases in FSH serum levels tightly correlate with increases in bone mass resulting from estrogen therapy [16].

FSH can affect the formation and activity of the bone resorbing cells, the osteoclasts (OCs), which differentiate from monocytes–macrophage precursors under the influence of macrophage-colony stimulating factor (M-CSF) and Receptor activator of nuclear factor kappa-B ligand (RANKL) expressed on osteoblasts and osteocytes [17]. In particular, FSH regulates osteoclastogenesis directly by binding to FSH receptor expressed on monocytes and OCs [18], and indirectly by enhancing the production of the pro-osteoclastogenic cytokine TNF-α from bone marrow macrophages/granulocytes and T-cells [19].

This study aimed to investigate cellular mechanisms underlying bone fragility in Turner's syndrome before induction of puberty and after HRT. For this purpose, we have evaluated the osteoclastogenic potential of peripheral blood mononuclear cells (PBMCs) belonging to girls with Turner's syndrome who had not been treated with HRT yet, hence showing high FSH serum levels, girls and young women on HRT who had normal serum FSH levels, and age-matched controls.

2. Patients and methods

2.1. Subjects

We recruited sixty Caucasian girls and young women with Turner's syndrome who referred to the Endocrinology Unit of the Pediatric Clinic of University of Bari and Messina, between March 2013 and April 2014, belonging to different age groups: 14 aged 1.5 to 10 years (mean age 5.94 ± 3.27), 20 aged 10 to 16 years (mean age 13.51 ± 2.06), and 26 young women (mean age 23.45 ± 6.80). Twelve girls out of 20 aged 10 to 16 years and all the young women were on HRT. For the puberty induction the patients with a chronological age of 11–12 years received transdermal administration of ethinyl estradiol at the dosage of 100 ng per kilogram per day for nine–twelve months, monitoring the response of breast development and endometrial thickness by ultrasonography. When the endometrium reaches 5 mm of thick was administered estrogen therapy in cycles of 25 days at the dosage of 0.01 or 0.02 mg/day as ethinyl estradiol or 1.25 mg/day of conjugated estrogens; in the last 11 days of the cycle was added medroxyprogesterone acetate at the dose of 10 mg/day.

None of them had history of fractures. Criteria of inclusion included a karyotype diagnosis of Turner's syndrome (without Y-chromosome material), and adequate thyroid function for at least 3 months in patients with hypothyroidism. Exclusion criteria included signs of spontaneous puberty (Tanner breast stage B2) for the group of girls, and personal history of spontaneous onset of puberty for the young women; use of medications, such as corticosteroids, presence of chronic disease, such as diabetes mellitus, renal failure, celiac disease, calcium and/or vitamin supplementation.

The control group was recruited on a voluntary basis in the outpatient clinic and included sex- as well as age-matched subjects referred to our hospital for minor surgery or electrocardiographic screening, and their mothers.

From patients and controls, after an overnight fast, venous peripheral blood samples were taken in opportune tubes for serum separation as well as in EDTA tubes for cell cultures and flow cytometry. For serum separation, the samples were opportunely centrifuged and immediately frozen at −80 °C until the determination was performed.

The work was approved by Messina and Bari University Hospital Ethical Committee, and written informed consent was obtained from the patients' parents or guardians, and from the patients when appropriate. The study was performed according to the Declaration of Helsinki.

2.2. Auxological and biochemical assessments

Girls and young women with Turner's syndrome underwent anthropometric measures (height, weight, body mass index-BMI). Pubertal development was evaluated according to Tanner [20]. Biochemical evaluation of FSH, LH and 17β−estradiol serum levels was performed using commercial EIA kits (Diagnostic System Laboratories, Inc., Webster, TX). Blood sampling was performed when the patients were at follicular phase of cycle.

25-OH Vitamin D serum concentration was measured by immunological tests based on the principle of chemiluminescence (CLIA) (Liaison assay; DiaSorin, Stillwater, MN). Osteocalcin serum concentration was measured by Enzyme Immuno Assay, using commercial kit (IBL, International, Germany). Calcium, phosphorus and alkaline phosphatase serum concentrations were measured by nephelometric method.

RANKL, osteoprotegerin (OPG), C-terminal telopeptide of collagen type 1 (CTX) (Biomedica, Vienna, Austria) and TNF-α (R&D Systems) were measured in the culture media and/or sera, using commercially available ELISA kits according to the manufacturer's instructions. The absorption was determined with an ELISA reader (550 Microplate Reader; Bio-Rad). The intra- and inter-assay coefficient of variation was <5% for all assays.

2.3. Bone mineral measurements

Bone mineralization was measured at lumbar spine L2–L4 by dual-energy X-ray absorptiometry (DPX) (ACN Unigamma X-Ray Plus; L'ACN Scientific Laboratories) and converted to SD scores (Z-scores) in relation to age and sex-matched normal population. Height also was measured at the time of BMD evaluation.

To minimize the effect of body size on areal BMD results at the lumbar spine, a validated transformation was made of the DXA data to calculate a volumetric density (bone mineral apparent density [BMAD]). This uses the assumption that the measured site is a cylinder with a volume proportional to the second power of the projected anteroposterior area obtained from DXA measurement of areal BMAD [21]. BMAD of the spine is calculated as BMC (L1 − L4) / projected area^2. The Z-score values for height and for BMAD were calculated by subtracting the corresponding age- and sex matched values and dividing by the corresponding standard deviation. The same correction was applied for controls.

2.4. Cells and culture conditions

OCs were obtained from non-fractioned and T-cell-depleted PBMCs of subjects with Turner's syndrome and controls. PBMCs were isolated by centrifugation of peripheral blood samples over Histopaque 1077 density gradient (Sigma Chemical, St. Louis, MO), diluted at 1×10^6 cells/ml in α-MEM (Invitrogen, Paisley, UK) and supplemented with