Gene study (OPG, RANKL, Runx2 and AGE receptors) in human osteoblast cultures from patients with type 2 diabetes mellitus and hip fracture. Influence of levels of glucose and AGEs

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Summary

Introduction: Diabetes mellitus (DM) type 2 is associated with a higher risk of osteoporotic fracture. Many factors have been indicated as possible mechanisms responsible for this, among which are changes in bone remodelling which may be induced by variations in circulating glucose or by the presence of non-oxidative advanced glycosylation end products (AGEs). The aim of this work has been to evaluate whether these variations generate changes in the expression of genes related to osteoblast differentiation and activity (OPG, RANKL, Runx2 and AGER) in primary cultures of human osteoblasts (hOB).

Material and methods: 12 patients were studied, belonging to three groups: 4 with osteoporotic fracture, 4 with osteoporotic fracture and DM type 2, and 4 patients with osteoarthritis, but who were not osteoporotic or diabetic (control group), with an average age of 80 ± 8, 84 ± 10 and 66 ± 11 years, respectively. Primary cultures of hOB from trabecular bone were carried out, to which were applied different stimuli over 24 hours. The gene study was carried out using real-time PCR.

Results: The genetic expression of RANKL was seen to increase in the diabetic group, although not to a significant degree, in the cultures which were high in glucose and high in glucose supplemented by AGEs (1.9 and 4.6 times higher vs control conditions; 2.3 and 4.4 times vs control group, respectively). The RANKL/OPG ratio stayed constant in the control group, however, in the diabetic group an increase was seen in all experimental conditions. In the case of Runx2 we found a significant increase in expression in the diabetic group with respect to the control group in the culture high in glucose and AGEs (OA = 1.08 ± 0.43; OP+DM = 3.33 ± 0.73; p = 0.039). No significant changes in the expression of OPG and AGER with respect to the control condition were observed for any of the culture conditions, in any of the patient groups.

Conclusions: The presence of a hyperglycaemic environment and AGEs alters the genetic expression of RANKL, of the RANKL/OPG ratio and Runx2 in osteoblast cultures from diabetic patients with hip fractures. These variations could generate changes in bone remodelling which could explain, at least partly, the lower bone resistance and the increase in the incidence of non-traumatic fractures in these patients.

Key words: osteoporosis, fracture, diabetes mellitus, osteoblasts.
Introduction
Osteoporotic fractures have a high prevalence in developed countries. Among these fractures, those of the hip are the most devastating due to their high mortality and the low number of patients who manage to recover a sufficient degree of functional activity to allow them to be independent. Diabetes mellitus (DM) is a metabolic disease which is also common in the population, with high mortality and morbidity, whose prevalence increases with age, as is the case with osteoporosis.

In patients with DM type 2 (DM2) it has been possible to confirm that, in spite of their having increased levels of bone mass, there is an incidence of osteoporotic fractures 2.8 times higher than in the general population, it being postulated that the disease itself, or the complications which originate from it, may alter skeletal bone remodelling, affecting bone formation and/or resorption, and with this, bone resistance. Among those mechanisms which are considered to be implicated in this lower bone resistance are included: a deficit or resistance to insulin, the hyperglycaemia to which the bone and the microenvironment of the bone medulla are subject, the higher concentrations of the advanced glycation end products (AGEs) and their effects on the proteins of the bone matrix, the alteration in production of adipokines and cytokines and its negative effects in the bone cells and, finally, the damage that the neuromuscular system may exert on the skeleton, leading to a greater propensity to falls in these patients. In spite of the fact that there are many factors postulated, there are few studies which analyse the importance of each of them, or the mechanism acting intimately on the deterioration of bone metabolism.

In the process of bone formation the signals which determine differentiation, replication and survival of the osteoblast cells will be critical for correct bone metabolism. Among these signals will be determinant genes included in the OPG/RANK/RANKL system, others such as Runx2, and maybe also those responsible for non-oxidising advanced glycation end product receptors (AGER). In DM the number and activity of bone-forming cells may be altered, as well as the response of these cells to local or systemic factors which contribute to bone remodelling.

OPG/RANK/RANKL is the main system of communication between osteoclast and osteoblast line cells, through which most systemic medicines, cytokines and growth factors which have an influence on bone remodelling work. Runx2 is one of the multifunctional transcription factors which controls the development of the skeleton through the regulation of the differentiation of chondrocytes and osteoblasts, directing the multipotential mesenchymal cells towards the osteoblast cell line and triggering the expression of most of the genes which code for the proteins of the extracellular matrix. Runx2−/− mice show a total lack of bone from birth.

The advanced glycation end product receptors (AGER) bond with a wide variety of structural and functionally related ligands, including the AGEs, such as pentosidine and carboxymethyl-lysine. The combination of AGEs -AGER promotes an overexpression of AGER, resulting in a permanent state of cellular activation, which it is thought contributes to the pathology of chronic disorders such as diabetes. The AGEs form slowly with age in response to physiological levels of sugars, as well as being increased in hyperglycaemic environments, as is the case with diabetes, which is also associated with chronic inflammatory complications. They combine with the membrane receptors (AGER) on the surface of the osteoblast line cells triggering intracellular signals which result in responses such as the expression of RANKL, the promotion of osteoblast differentiation and activation, and with this, bone resorption, as well as inducing osteoblast apoptosis.

The influence of hyperglycaemia or AGEs on the expression of genes related to bone metabolism has been studied before in animal models, in cell lines, and in primary cultures of osteoblasts from patients with arthritis, but not with diabetic disease, as is the case with this study.

Given that to date there is very little knowledge about the influence of DM2 on bone metabolism and that it is not known how high levels of glucose and AGEs may influence osteoforming cells and the expression of these genes (OPG, RANKL, Runx2 and AGER), we have proposed this study, which has as its main objective the analysis of these aspects in patients with DM2 and non-traumatic hip fracture.

Material and methods
Subjects of the study
We included 12 patients belonging to three study groups: 4 patients with non-traumatic hip fracture and DM2 (OP+DM group formed of 4 women), 4 patients with non-traumatic hip fracture without DM (OP group formed of 3 women and 1 man), and 4 patients subject to arthroplasty of the hip due to problems of osteoarthritis, without history of either osteoporosis or DM2, as a reference group (OA group formed of 3 women and 1 man).

The inclusion criteria for the OP+DM group was to have suffered from DM2 for a minimum of 5 years since diagnosis, as well as having a fragility fracture of the hip, due to a fall from a height lower than the height of the individual, without any acceleration mechanism. For the patients in group OP, to have had a hip fracture due to fragility without diabetic syndrome. And lastly, those in the OA reference group, not having previously been diagnosed with either diabetes or osteoporosis, nor having history of fragility fracture since the age of 50. The exclusion criteria for all the groups were the taking of medicines which have an influence on bone metabolism (corticoids, contraceptives, antiresorptives, immunosuppressors, glitazones) or having endocrine or systemic diseases with an influence on bone remodelling, as well as treatment for tumours in the last 10 years.
The sampling period was 6 months and all subjects came from the traumatology and orthopaedic service of the Virgin Macarena University Hospital in Seville. The patients were informed and gave their written consent, and the trial was approved by the ethics and health research committee of the hospital. In addition, the participants were subject to a questionnaire regarding their age, years of menopause in the women, toxic habits (alcohol and tobacco), semiquantitative consumption of calcium by means of a survey of daily intake of milk and milk-derived products (estimating each glass of milk or portion of cheese to be 200 mg of calcium), personal and family history of first degree fractures, chronic taking of medicines and concomitant diseases. For those patients in the OP+DM group, also included were the number of years the disease had been in development, hypoglycaemic treatments and presence/absence of chronic complications of the diabetic disease itself, such as retinopathy, nephropathy or arteriopathy. The height and weight of all patients was measured and their BMI calculated.

A blood sample was taken from all patients for the first 4 days after the episode of fracture, to determine the following blood biochemistry parameters: glucose, urea, creatinine, enzymes of hepatie function, total alkaline phosphatase (AP), calcium and phosphorus (Autoanalizer DAX-96), glycated haemoglobin (HbA1c) (HPLC); 250HD, hepatic function, total alkaline phosphatase (AP), meters: glucose, urea, creatinine, enzymes of determine the following blood biochemistry para-

The biopsies of femoral bone were processed in sterile conditions immediately after being extracted by surgery, followed by the carrying out of cell cultures, as will be detailed in the following section.

Cell cultures
We carried out primary cultures of human osteoblasts (hOB) from explants of trabecular bone of 1-2 mm, extracts from the femoral heads biopsied. These were rinsed with PBS and subsequently distributed in 90 mm Petri dishes at a ratio of 10-15 explants per dish, attempting to obtain 3 to 5 dishes for each subject.

They were incubated in DMEM medium (4.5 mM of glucose), supplemented with 10% foetal bovine serum (FBS), 0.5% fungicide, 1% L-glutamine, 1% Na-Pyr and 1% of antibiotic (100U/ml of penicillin and 100 μg/ml of streptomycin) at 37ºC and 5% CO2 for 7 days.

The culture medium was changed twice a week until subconfluence was reached. Once this moment had arrived (after between 4 to 6 weeks) we carried out a cell passage. We trypsinised (Trypsin-EDTA) and plated the cells (300,000 cells/well on dishes of 6 wells) in the same medium as already mentioned.

On reaching sub-confluence again, the cells were washed with saline PBS buffer and incubated for 24 hours with the same medium, without FBS to have the cultures at the same stage of growth at the start of the experiment. Different conditions of culture were established over 24 hours to evaluate the effect of high concentrations of glucose and AGEs on the functioning of the osteoblasts: a) medium low in glucose (4.5 mM), b) rich in glucose (25 mM), c) rich in glucose (25mM) supplemented with AGEs (0.1 mg/ml) (Advanced Glycation Endproduct-BSA, Calbiochem, USA and d) medium low in glucose supplemented with manitol (25 mM) to discount the possible effect of the hyperosmolarity which a high concentration of glucose could exert on osteoblasts in culture.

The following were analyzed in all cultures:
1. Cell viability, calculated with the TripanBlue exclusion test at 0.5%.
2. Bone alkaline phosphatase (BAP) activity, measured after incubation for 1 hour at 37ºC in 0.1 M of NaHCO3- Na2CO3 pH 10, 0.1% Triton X-100, 2 mM MgSO4 and 6 mM PNPP. The reaction was stopped with 1M of NaOH and the absorption measured at 405 nm. The percentage of changes in the BAP activity in relation to the value found in the control was calculated using the formula: M= absorbance value at 405 nm/absorbance value at 560 nm. The percentage change= (M of the control M-test )/M of the control x 100.

Quantification of the expression of mRNA (OPG, RANKL, Runx2 and AGER)
Using the cells gathered from each of the experimental culture conditions an extraction of total RNA was carried out (High Pure RNA Isolation. Roche, USA). The concentration of RNA was measured at 260 nm (GeneQuant, Amersham Biosciencies). Subsequently, the RNA obtained was retrotranscribed to cDNA (QuantiTec Reverse Transcription, Qiagen).

The analysis of the gene expression of the different genes of the study was carried out using PCR real time (QuantiTec SYBR Green PCR, Qiagen; Primers Applied Biosystem).

The results for each of the genes studied were referenced to those obtained for ribosomal gene 18S and in turn, with the control condition (4.5 mM glucose).

Statistical analysis of the results
For the statistical analysis of the results we used SPSS version 18.0. The individual results were reviewed to avoid the loss of data and unusual values. All the experiments were reproduced in duplicate and the descriptive statistical data of the numerical variables were expressed as mean ± standard deviation. First, the homogeneity of variance of the variables was analysed. In those cases in which homoscedasticity was confirmed an ANOVA test was applied with Tukey’s HSD post-hoc analysis. For those which showed heteroscedasticity, a Welch F test was applied with a Games-Howell post-hoc analysis. Correlation studies were made using the Pearson or Spearman correlation test depending on the normal distribution, or not, of the variables. In all cases a significance level of p<0.05 was required.
RESULTS

The results we present here are preliminary, taking into account the fact that only 4 subjects per study group were evaluated. The characteristics of the patients in the three groups studied, as well as the average values of the blood parameters analysed are found in Table 1.

The age of the patients in the OA group was significantly lower than the age of the other groups, which means that for the statistical comparison of the other parameters an adjustment for age was made. The BMI was lower, although not statistically different, in the OP group. The renal function was normal and comparable across the three groups.

The levels of glucose when fasting and HbA1c were in the normal range, without showing significant differences between the three groups, while the OP+DM patients were those who had the highest value, with HbA1c reaching levels 25% higher in this group, with respect to the other groups studied. In terms of the parameters related to calcium metabolism, only the levels of phosphorus and AP were significantly different when comparing the three groups, the levels of phosphorus being lower, and those of AP higher, in the OP group in relation to the OA and OP+DM groups. The rest of the parameters, while not showing significant differences, had some noteworthy aspects. The levels of vitamin D were, in all cases, below 20 ng/ml and somewhat lower in those patients who had had a hip fracture, with or without DM, with respect to the controls (average values of phosphorus being lower, and those of AP higher, in the OP group in relation to the OA and OP+DM groups. The rest of the parameters, while not showing significant differences, had some noteworthy aspects. The levels of vitamin D were, in all cases, below 20 ng/ml and somewhat lower in those patients who had had a hip fracture, with or without DM, with respect to the controls (average values of 9.6 ng/ml and 9.2 ng/ml vs 12.3 ng/ml, respectively).

The levels of glucose in the culture medium and the PTH were higher in the OP group, as well as the markers for bone remodelling, both for formation and resorption, P1NP and ß-CrossLaps.

The blood levels of IGF-1 were lower in the two groups with hip fracture (average values in OP = 25 ng/ml, in OP+DM = 37.7 ng/ml and in OA = 41.8 ng/ml), becoming significantly different in the OP group compared with the OA group (p=0.011).

CELL VIABILITY AND BAP

No significant differences were found in any of the groups for any of the conditions studied, with all the cultures having a viability higher than 85% and BAP staining higher than 95%.

RT-q-PCR

In the study of the expression of the osteogenic genes studied we found high levels of interpersonal variation.

The results of the expression of OPG are represented in Figure 1. None of the different culture conditions significantly influenced the gene expression of OPG in the three groups studied, nor was any difference seen between the three groups. Only the situation of hyperglycaemia and even more, the combination of hyperglycaemia and AGEs, had a higher expression of OPG in the OP group, reaching a level 1.7 times that of the control group.

The gene expression of RANKL (Figure 2) was seen to increase in the OP+DM group, although not significantly, with the expression higher in the condition high in glucose, both in respect of the control condition and the control group (1.9 and 2.3 times higher, respectively). The same occurs in the condition high in glucose supplemented with AGEs (4.6 times vs control condition and 4.4 times control group, respectively). The results show a decrease in the gene expression of RANKL in the presence of a high concentration of glucose, both in the control and in the OP group. However, in the presence of the AGEs, the expression was similar to the control condition in both groups.

The values of the RANKL/OPG ratio (Figure 3) remain constant in the control group. However, in the OP+DM group they are seen to be increased in all of the experimental conditions. With regard to the OP group a reduction occurs in the presence of high glucose, and an increase, similar to that we observed in the diabetic group, with high glucose plus AGEs.

In the case of the gene expression of Runx2 (Figure 4) we found a significant increase in the OP+DM group with respect to the control group in the experimental condition which combined high glucose and AGEs (OA = 1.08 ± 0.43; OP+DM = 3.53 ± 0.73; p=0.039). In this same condition we also observed an increase in the expression in the OP group, although it was not significant.

Lastly, the results of the gene expression of AGER (Figure 5) were very similar between the groups studied, being higher than in the control condition. However, these results were not significant in any of the groups and for none of the experimental conditions.

We didn't find any statistically significant correlation between the biochemical and anthropometric parameters (Table 1) and those genes involved in bone metabolism which were studied (Figures 1-5).

DISCUSSION

The most significant results of this study show us that the addition of glucose, at high concentrations, and above all the combination of glucose and AGEs, to cultures of hOB from patients with DM2 and hip fracture increases the expression of the RANKL genes, the RANKL/OPG and Runx2 ratio, compared with a person with neither hip fracture or DM.

In agreement with our results, Li et al. demonstrated in studies in rats that high levels of glucose in the culture medium induce higher osteoblast differentiation in ligament cells stimulated to become osteoblasts, through a significant increase in levels of gene expression for Runx2. However, other authors have described other different results depending on the type of cells used, the levels of glucose in the culture medium and the time exposed to it, these factors not being consistent.

It has been possible to show that transgenic mice which overexpress Runx2 also have an incre-
Ase in the expression of RANKL, giving as a result mice with a significant loss of bone mass. In this case the authors associate these findings with the blocking of osteoblast differentiation, which is to say, that the overexpression of Runx2 is related to a greater number of less differentiated osteoblasts which express a greater quantity of RANKL, activating osteoclastogenesis and thus generating a greater number of mature osteoclasts. The presence of high blood concentrations of glucose and AGEs are normally very frequent metabolic changes in diabetic patients, as is apparent from our results, causing an overexpression of Runx2 in osteoblast cells, as well in as the expression of the RANKL/OPG ratio, which may result in an increased rate of remodelling and have a negative impact on bone resistance.

We found in our study an increase in RANKL and the RANKL/OPG ratio, highest above all in the OP+DM group. De Amorim et al. found that in diabetic rats with tibial fracture there is an increase in the RANKL/OPG ratio with respect to healthy rats, which supports our results.

What we find interesting is that the osteoblasts from patients with hip fracture, with or without DM2, are those which have a higher variability in the expression of the genes in the study resulting from the stimulus taking place in the different situations of the in vitro cultures. Thus, in earlier research carried out by our group in cultures of hOB from patients with hip fracture as against patients with arthrosis, we also confirmed that those patients with osteoporosis were those who had the greatest modifications in gene expression under the different stimuli. This may indicate that, in vivo, these cells of osteoporotic patients are already conditioned by the environment in which they are found, to response levels higher than in an individual with healthy bone metabolism. Equally, those patients with DM2 with altered bone metabolism induced by hyperglycaemia and habitual oxidative stress, are also those who respond more in the in vitro conditions with which we have experimented.

The gene expression of AGER is seen both in the high glucose condition and in glucose supplemented with AGEs in the control (OA) and osteoporotic (OP) groups, but not in the diabetic group (OP+DM), which are similar to the baseline condition. These results coincide with those obtained in MC3T3E1 cells by Mercer et al. and in human osteoblasts by Franke et al., where they analysed the expression of AGER in cultures exposed to AGEs. In this case, it is suggested that as a consequence of the presence of a higher concentration of AGEs a greater activation of AGE-AGER is produced, which would cause osteoblast dysfunction. The results, with regard to the expression of AGER, in the OP+DM group indicate that the osteoblasts in these patients may have suffered some kind of habituation to high extracellular levels of glucose and AGEs, which makes them not react to

| Table 1. Anthropometric characteristics and blood biochemistry parameters of the groups studied |
|--------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Age (years)                                      | 66 ± 11 *                       | 80 ± 8                          | 84 ± 10                         | *p=0.04                          |
| BMI (kg/m²)                                      | 30.8 ± 2.8                      | 23.6 ± 2.3                      | 31.5 ± 4.9                      |                                  |
| Creatinine (mg/ml)                              | 0.8 ± 0.05                      | 0.9 ± 0.05                      | 0.8 ± 0.06                      |                                  |
| Glucose (mg/dl)                                 | 84.8 ± 4.9                      | 97.5 ± 0.5                      | 100.7 ± 12.9                    |                                  |
| HbA1c (%)                                       | 5.1 ± 0.3                       | 5.1 ± 0.5                       | 6.5 ± 0.6                       |                                  |
| 25(OH)D (ng/ml)                                 | 12.3 ± 3.1                      | 9.2 ± 3.2                       | 9.6 ± 0.7                       |                                  |
| PTH (pg/ml)                                     | 48.1 ± 10.6                     | 72 ± 55                         | 55 ± 4.9                        |                                  |
| PIPN (ng/ml)                                    | 38.0 ± 16.2                     | 42.5 ± 1.2                      | 67 ± 20.3                       |                                  |
| β-CrossLaps (ng/ml)                             | 0.45 ± 0.09                     | 0.83 ± 0.07                     | 0.62 ± 0.19                     |                                  |
| Phosphorus (mg/dl)                              | 3.3 ± 0.1                       | 2 ± 0.3 *                       | 2.5 ± 0.3#                     | *p=0.004, #p=0.022               |
| AP (U/L)                                        | 160.3 ± 16.3                    | 247.5 ± 25.5 *                  | 181 ± 39.4                      | *p=0.04                          |
| Ca corrected (mg/dl)                            | 9.6 ± 0.2                       | 9 ± 0.1                         | 9.5 ± 0.3                       |                                  |
| IGF-1 (ng/ml)                                   | 41.8 ± 2.9                      | 25 ± 0.01 *                     | 37.7 ± 12.7                     | *p=0.011                          |

*OA vs OP; # OP vs OP+DM
these stimuli, at least during the first 24 hours of treatment. The interaction of AGEs with their receptors changes cell signalling, promotes an increase in the generation of reactive oxygen species (ROS), with the consequent oxidative stress. In turn, the long term hyperglycaemic environment, such as occurs in diabetes, increases the production of AGEs and of ROS, which may cause a decrease in the proliferation, and an increase in the apoptosis, of osteoblasts.

We have been able to confirm that those patients with hip fracture without DM have high levels of PTH and low levels of IGF-1, both conditions being associated with senile osteoporosis and hip fracture. It is known that with age there are lower levels of vitamin D, as a consequence of a lower provision, lower absorption and less exposure to sun, which results in secondary hyperparathyroidism, with the consequent increase in remodelled bone and a higher risk of suffering fractures.

In people of an advanced age low levels of blood IGF-1 are also described which is correlated with low levels of bone mineral density and with an increased risk of fracture.

While it is true that most of the results we found may not have been statistically different due to the small sample size, we also have to take into account that the group of patients with DM2 had been developing the disease over a short period (average 5 years) and with highly adequate metabolic control (HBA1c average of 6.5%). As is known, the complications of diabetes are more acute both the longer its period of development and the more altered its carbohydrate metabolism is. Among the limitations of this study the most significant is the fact that it was not possible to count on a group of truly healthy people from who we could obtain bone biopsies, having to use as reference people with arthrosis, who in general and given the nature of this pathology, are always going to be younger than patients with hip fracture, bearing in mind that age is one of the independent factors with most influence on changes in bone remodelling. Finally, it would be a good idea to analyse whether or not the alterations found in genes have repercussions at the level of proteins.

In conclusion, and in view of our results, we are able to say that the presence of hyperglycaemia and AGEs alter the gene expression for RANKL, the RANKL/OPG ratio and Runx2 in osteoblast cultures from patients with osteoporotic fracture and in diabetics with this type of fracture, especially in the latter. This may generate alterations in bone remodelling (higher levels of β-Crosslaps and PINP) which may explain, at least in part, the lower bone resistance and the increase in the incidence of non-traumatic fracture in these patients.
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