INTRODUCTION:
Fibrin sealants have been used for cell delivery and shown to be a suitable delivery vehicle for growth factors (1,2). Although no direct evidence of osteoinductivity has been reported when fibrin sealant is implanted alone (1,3), several in vitro studies reported an increase in bone formation when using fibrin sealant seeded with progenitor cells or in combination with other biomaterials (4,5). However, because these studies utilized different sources of fibrin sealants and in vivo models, it is difficult to identify the effect of fibrin sealant on the seeded or surrounding progenitor cell behavior and ultimately on bone regeneration.

The possibility exists that the compositional ratio of fibrinogen complex (FC) and thrombin, the two plasma-derived components mainly constituting fibrin, governs the fibrin microenvironment. We previously demonstrated the dependence of human mesenchymal stem cells (HMSC) morphology and proliferation on the differentiation. We previously demonstrated that when using a formulation of Tissuele® fibrin sealant containing a high concentration of FC, ALP activity increased, small nodules of bone mineralization appeared, and ALP, BSP and OPN gene expression were upregulated. These results suggest that HMSC were undergoing differentiation towards the osteogenic phenotype, possibly because of the presence of growth factors (especially TGF-β1 and b-FGF) in this fibrin sealant. However, the low number and the small size of the mineralization nodules as well as the absence of OCN upregulation even after 28 days of incubation suggest that HMSC did not undergo full osteogenic differentiation when seeded in the fibrin gels. Moreover, levels of osteogenic gene expression remained at lower levels than those measured in mature osteoblasts.

MATERIALS AND METHODS:
Eight different formulations of fibrin gels (Tissuele™, Baxter) were prepared using different concentrations of FC and thrombin, from 5-50 mg/ml and 1-250 U/ml, respectively (final concentrations in the gels). HMSC (Poietics™, Cambrex) were resuspended in the FC component of the fibrin gel and added to 24-well plates (8000 cells per well). Volumes of thrombin were then added to allow the formation of fibrin clots for 2 hours before adding growth medium (1 ml per well) with no supplement of osteogenic factors. Plates were incubated for up to 28 days, and medium was changed every 2-3 days.

A standard alkaline phosphatase (ALP) assay was first performed to determine HMSC osteogenic differentiation potential in each formulation of the fibrin gels at days 0, 3, 7, 14, 21 and 28. Von Kossa staining was then used to assess bone mineralization after 21 and 28 days (gels were observed under light microscopy at a magnification of 100x) and the relative level of osteogenic gene expression (ALP, bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN)), compared to 18S, was also analyzed by real time RT-PCR at days 0, 7, 14 and 28, in a fibrin gel formulation that induced the highest ALP activity.

In order to account for cell response variations within patients, cells from 2 different lots were used for the real time RT-PCR analysis. Statistical analysis was performed using the ANOVA test with p<0.05.

RESULTS:
Results showed that ALP activity started to be significant at day 14 and reached maximum at day 28. Higher concentrations of FC induced higher ALP expression, suggesting a higher differentiation rate. Thrombin concentration had a lesser effect.

Von Kossa staining in the fibrin gel formulation containing 34 mg/ml FC (i.e. a high concentration of FC) and 1 U/ml thrombin showed the presence of relatively small nodules of calcium phosphate after 21 and 28 days of incubation (Fig 1). The nodules appeared in the areas with cells, and mainly in the areas with holes in the gels.

Real time RT-PCR analysis using the same fibrin gel formulation as for Von Kossa staining showed that results obtained with two different lots followed the same tendencies, although one lot gave higher upregulation of ALP and BSP and a lower upregulation of OPN than the other lot. ALP gene expression was upregulated between day 7 and day 28 (7.4 fold for lot #1 and 6.8 fold for lot #2). Also, an upregulation of up to 22.7 fold in the level of BSP (16.6 for lot #1 and 22.7 for lot #2) and up to 4.9 fold in the level of OPN gene expression (4.9 for lot #1 and 3.6 for lot #2) was detected at day 28 compared to the levels of expression at day 0 (Fig 3 and 4, respectively).

Surprisingly, the expression level of OCN did not increase and was not significantly different after 28 days of incubation (p>0.05).

DISCUSSION:
The present study demonstrated that, when using a formulation of Tissuele™ fibrin sealant containing a high concentration of FC, ALP activity increased, small nodules of bone mineralization appeared, and ALP, BSP and OPN gene expression were upregulated. These results suggest that HMSC were undergoing differentiation towards the osteogenic phenotype, possibly because of the presence of growth factors (especially TGF-β1 and b-FGF) in this fibrin sealant. However, the low number and the small size of the mineralization nodules as well as the absence of OCN upregulation even after 28 days of incubation suggest that HMSC did not undergo full osteogenic differentiation when seeded in the fibrin gels. Moreover, levels of osteogenic gene expression remained at lower levels than those measured in mature osteoblasts.

Von Kossa staining demonstrated relatively few and small nodules of calcium phosphate deposition, associated with the presence of cells and mostly in the regions of the clots depicting a partial dissolution of the gel (holes). The low number of nodules observed in the clots could be due to the low number of cells initially seeded in the gel (8000 cells) and the relatively large volume of growth medium used in the present experiment. Indeed, Jaiswal et al. (8) reported that the formation of mineralized matrix by HMSC was largely influenced by the initial cell-seeding density and the growth medium volume used for the cell culture. As for the results with Von Kossa staining, it is also possible that an initial higher cell-seeding density would increase the level of osteogenic gene expression, since osteogenesis was demonstrated to be triggered by higher number of cells (8).

Overall, the present study demonstrated that a formulation of Tissuele™ fibrin sealant containing a high concentration of FC may support HMSC differentiation into osteoblasts in vitro, but also suggest that the cells may not fully differentiate into mature osteoblasts in vitro. Further studies using different fibrin formulations in combination with synthetic materials and/or additional growth factors could therefore be conducted in order to allow a complete differentiation of HMSC in this microenvironment.

REFERENCES:

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