MMP-2, MMP-9 and their inhibitors TIMP-2 and TIMP-1 production by human monocytes in vitro in the presence of different forms of hydroxyapatite particles.

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MMP-2, MMP-9 and their inhibitors TIMP-2 and TIMP-1 production by human monocytes *in vitro* in presence of differents hydroxyapatite : importance of particle physical characteristics

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Abstract

After calcium-phosphates biomaterials based implantation like hydroxyapatite coating, particles are released in the periprosthetic tissues. Fibrous tissue around wear debris are mainly made of monocytes that can be produced metalloprotease (MMP), considered to be the key enzymes in extracellular matrix turnover. Tissue inhibitors of metalloproteinases are important regulator of MMPs activity. Interleukin–1 mainly produced by monocytes can also regulate MMPs production. In the present work, we have evaluated the effect of hydroxyapatite particles characteristics (size, shape and sintering temperature) on the MMP-2, -9 and their inhibitors TIMP-2, -1 production. Our results demonstrate that sintering temperature (that modify crystal size and surface area) have few effect on MMPs and TIMPs production. Not-phagocytable particles induced more MMP-9, altough phagocytable particles induced more IL-1β production. The shape of the particles was the most important factor since needle particles induced the most important quantity of MMPs and IL-1β.

Key-words : matrix metalloprotease, tissue inhibitors of metalloprotease, hydroxyapatite particles, human monocytes, physical characteristics.

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Introduction

Loosening of total joint replacement appears to result in part from the production and accumulation of particles at the prosthetic interface and the subsequent biological reaction [1-2]. Although metallic and polyethylene particles have been studied[3], little is known about the cellular response to hydroxyapatite (HA) despite the wide use of these materials[4]. Hydroxyapatite has been demonstrated to produce particles even when dense[5]. It has been demonstrated that the physical characteristics of the HA particles (size, shape and sintering temperature) could modify the toxicity of the biomaterials and the cytokines produced by various cell type[6-10].

Monocytes/Macrophages were commonly observed in the interface tissue, they are among the first cells to colonize the inflammatory site[11-13] and they play a key role in the immune response. Moreover, monocytes have been shown to produce metalloprotease at the prosthetic interface[14]. Matrix Metalloproteases (MMPs) are a family of proteolytic enzymes, are capable of degrading all major components of the extracellular matrix[15]. Their activities can be regulated by natural specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). A balance between MMPs and TIMPs is necessary for many physiological processes, and it has been suggested that imbalance may lead to the destruction of connective tissue, which occurs in a number of pathological events, including osteoarthritis and rheumatoid arthritis[16-17]. MMPs play an important role in the development of osteolysis and implant loosening, they can be produced after exposure to particles and they can be regulate by cytokines such as Interleukin-1 beta (IL-1β) an osteoclastic bone resorption stimulator[18-19].

Because particle-induced macrophage MMPs expression may increase tissue degradation at the bone-implant interface, we have studied the effect of the physical characteristics of hydroxyapatite (sintering temperature, size and shape) on the production of two MMPs :

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MMP-2 and MMP-9 and their respective inhibitors TIMP-2 and TIMP-1. We have also paid attention on the production of IL-1β by monocytes and its effect on MMPs production.

Materials and methods

Cells culture

Elutriated monocytes were used to evaluate MMPs, TIMPs and IL-1β production. Cells were obtained as described previously[20]. Briefly, monocytes from healthy consenting donors were collected by leukapheresis and purified by counterflow elutriation. Purity, after CD14 staining was, at least 95%, the rest being neutrophils. The human fibrosarcoma HT-1080 cell line (CCL-121) was used as an internal control for MMPs expression[21]. Cells were maintained in UltraCULTURE medium (Biowhittaker, Emerainville, France) supplemented with 2 mM L-glutamine, penicillin (5000 $U.ml^{-1}$) and streptomycin (25 $\mu g.ml^{-1}$) at 37°C in a saturated 5% CO_2 95% air atmosphere. The cell density was 2.10^5 $cells.ml^{-1}$ in a 5-ml total volume. Cells of each donor were exposed or not (control cells) during 6, 18 or 24 hours to the different particles described above. A positive control was made by exposing cells to zymosan particles. Zymosan particles were chosen because of their inflammatory actions on cells[22]. Conditioned media and cells were harvested from these cultures and kept frozen at –20°C until use.

The viability of cells was evaluated by trypan blue exclusion test. The viability was >95% in all of the experiments (before and after exposure to particles).

Particles characteristics

Six hydroxyapatite based powders were studied. They differed by their size range, shape and surface area. For all powders, the increase of the sintering temperature increased the crystal size and decreased the specific surface. It was impossible to obtained not sintered spherical particles, needle shape particles sintered at 1180°C and large needle particles. The particles characteristics were already published[23]. The powders were constituted of hydroxyapatite particles of 99% purity (Urodelia, Saint Lys, France). In resume, three hydroxyapatite powders had a spherical shape : their size range was between 1 to 30 µm (sintered at 600°C or 1180°C) or 170 to more than 300 µm, the last powders was sintered at 1180°C. Two other hydroxyapatite powders had irregular shapes with a distribution size between 1 to 30 µm (sintered at 600°C or 1180°C). One hydroxyapatite powders had a needle shape, a size range between 1 to 30 µm and was sintered at 600°C.

All powders characteristics are reported in table 1.

The human monocytes were cultured with the HA powder with the surface area ratio equal to 1 (SAR = surface area of cell / surface area of material). Previous studies demonstrated that cells were sensitive to the surface of material present in the cell culture[10,24-25].

HA powders used in the culture were treated to remove surface endotoxin. The powders were baked to 110°C for 12 h and used in aseptic conditions in a Class II safety cabinet.

Analysis of cytokines steady-state mRNAs level by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared with TRIZOL reagent (Life Technologies, France). RNA extraction was performed using phenol/chloroform extraction followed by ethanol precipitation. RNA (0.4 µg) from each sample was reverse-transcript with oligo-dT as the first-strand cDNA primer and Moloney murine leukaemia virus reverse transcriptase σM-MLV RT superscript

(Life Technologies, France) as described previously by Grandjean-Laquerriere et al.[23]. Primer sequences for MMP-2, MMP-9, TIMP-1, TIMP-2, IL-1β and for the internal control β-actin were described in Table 2. Reverse transcribed RNA were subjected to PCR using following reactions: 95°C during 3 min; 30 cycles of 94°C during 1 min, 68°C (MMPs, TIMPs and IL-1β) or 58°C (β-actin) during 1 min; 72°C during 1 min, and 72°C of 7 min for final extension. Number of cycles for MMP-2, MMP-9, TIMP-1, TIMP-2, IL-1β and β-actin was 39; 39; 23; 23; 21 and 25, respectively. The reagents and the classical PCR conditions have been optimised. The amplification products were separated electrophoretically on 2% agarose gels with ethidium bromide and analysed by the imager analyser BIORAD Fluor-S.

We showed previously that this RT-PCR technique is highly reproducible[26-28].

Gelatin zymography

Gelatinolytic activities in conditioned media and cellular extracts were performed according to Devy et al.[29]. Cell culture supernatants were harvested after 6, 18 and 24 hours of culture in Ultraculture medium in the presence or absence of hydroxyapatite particles or zymosan particles. Cellular extracts were obtained after lysis in 0.1 M Tris-HCL pH 8.1, and 0.4 % Triton X-100. An appropriate volume of conditioned media or cellular extract corresponding to an equal amount of cells (2.10^5 cells) was applied to 10% SDS-polyacrylamide gels containing 1 $mg.ml^{-1}$ gelatin (Sigma). After electrophoresis, SDS was removed from the gel by two incubations in 2% Triton X-100 for 30 min. After overnight incubation at 37°C in TCS buffer (50 mM Tris-HCl pH 7.4, 0.2 M NaCl and 5 mM CaCl2), the gels were stained for 90 min with Coomassie Blue. Proteolytic activities were evidenced as clear bands against the blue background of stained gelatin. Gels were analysed by the imager analyser BIORAD Fluor-S.

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Results

MMPs expression

To study the HA-mediated influence on MMP-2 and MMP-9 expression by human monocytes, cells were cultured in the presence of 6 different HA powders or zymosan particles. Following 6 h and 18 h after incubation, cells were harvested and MMP-2 and MMP-9 mRNAs steady-state levels were determined by RT-PCR analysis. As presented in figure 1, cells expressed constitutively low levels of MMP-2 and MMP-9 mRNAs. After 6 hours of cell culture, we observed an increase of both MMP-2 and MMP-9 mRNAs expression for all HA powders tested and for the inflammatory control, zymosan particles (Figure 1A and 1B). This expression was quite the same whatever the physical characteristics of the particles.

After 18 hours of cell culture, we obtained differences between MMP-2 and MMP-9 mRNAs expressions (Figure 1A and 1B), the mRNAs expressions were different in function of the physical characteristics of the particles. Indeed, the levels of MMP-2 mRNAs in presence of HA 1 to 5 were the same as the control and we showed a decrease of MMP-2 mRNA expression in presence of the needle particles (HA6) and zymosan particles (Figure 1A and 1Ba).

As shown in figure 1A and 1Ab the levels of MMP-9 mRNAs in presence of spherical particles (HA1, HA2, and HA3), irregular shape particles (HA4, and HA5) and zymosan particles were close to the control one whereas expression of MMP-9 mRNA strongly increase with needle particles (HA6) (Figure 1A and 1Ab).

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The sintering temperature has few effects on MMP-2 and MMP-9 mRNAs expression. But the size and the shape of the particles modify the expression of the MMPs studied.

MMPs activities by human monocytes exposed to HA or zymosan particles

To determine if the modulation of MMP-2 and MMP-9 mRNA expression by HA in human monocytes was associated with MMP-2 and MMP-9 gelatinolytic activity, conditioned culture supernatants and cellular extracts were analyzed by zymography.

Monocytes exposed to HA- or zymosan are able to stimulate MMP-2 mRNA expression, but no gelatinolytic activity could be detected whatever time of incubation both in conditioned culture supernatants and cellular extracts (Figure 2 and 3). In other conditions, it has already been observed that monocytes produced few or no pro-MMP-2 or MMP-2[30].

On the other hand, we observed, in the conditioned culture supernatants, the presence of pro-MMP-9 and MMP-9 gelatinolytic activity (Figure 2 and 3). We showed only the presence of pro-MMP-9 gelatinolytic activity with monocytes alone (Figure 2 and 3). After 6 hours of incubation, we obtained an increase of the pro-MMP-9 gelatinolytic activity in the culture media when cells were cultured with zymosan particles and all of the hydroxyapatite particles (Figure 2).

After 18 hours of cell culture, we showed a different profile of the pro-MMP-9 gelatinolytic activity. Indeed, in presence of phagocytable spherical particles (HA1, and HA2) and indifferent shape particles (HA4, and HA5), a diminution of the activity was shown (figure 2). But gelatinolytic activity was close to the control one when cells were exposed to non-phagocytable spherical particles (HA3), needle particles (HA6), and zymosan particles.

After 24 hours of cell culture, we showed few differences of pro-MMP-9 gelatinolytic activity in relation to 18 hours of cell culture (figure 2). We showed still a decrease of pro-MMP-9

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gelatinolytic activity in presence of phagocytable spherical particles (HA1, and HA2), indifferent shape particles (HA4, and HA5), and zymosan particles, and an activity close to the control one with non-phagocytable spherical particles (HA3), and needle particles (HA6).

In the cellular extracts, after 6 hours of cell culture, the pro-MMP-9 gelatinolytic activity decreased when cells were exposed to all HA particles and zymosan particles and no active form of the MMP-9 was detected (Figure 3).

After 18 hours of cell culture, we obtained a decrease of the presence of pro-MMP-9 gelatinolytic activity using all HA particles and a quasi-complete disappearance of pro-MMP-9 gelatinolytic activity when cells were exposed to zymosan particles (figure 3Ba). There was still no active MMP-9 gelatinolytic activity when cells were cultured alone and with zymosan particles, phagocytable spherical particles (HA2) or non-phagocytable spherical particles (HA3). On the other hand, an active form of the MMP-9 gelatinolytic activity was detected using phagocytable spherical particles (HA1), indifferent shape particles (HA4, and HA5) and needle particles (HA6) (Figure 3Bb).

After 24 hours of cell culture, the pro-MMP-9 gelatinolytic activity still decreased when cells were exposed to phagocytable spherical particles (HA2) and zymosan particles (figure 3Ba). This activity was close to the control one using phagocytable spherical particles (HA1), non-phagocytable spherical particles (HA3), indifferent shape particles (HA4, and HA5) and needle particles (HA6). The gelatinolytic activity of the cellular extract of active MMP-9 was still inexistent in the control whereas it was present with all HA particles and zymosan particles (figure 3c). The gelatinolytic activity of active MMP-9 appeared at this time using zymosan particles, and spherical particles (HA2, and HA3). When cells were cultured with phagocytable spherical particles (HA1), indifferent shape particles (HA4, and HA5) and needle particles (HA6), the activity of active MMP-9 increased compared to 18 hours.

We obtained an effect of the sintering temperature on the activity of MMP-9 only using spherical particles. There was few effect of the size of the particles on the activity of MMP-9. The most important modification of the activity of MMP-9 was obtained using different shaped particles.

TIMPs expression by human monocytes exposed to HA or zymosan particles

To study the down-regulation of MMP-2 and -9, TIMP-2 and TIMP-1 expression was study when cells were exposed to HA particles. Production of TIMPs is important in the control of MMPs activity and therefore we wanted to establish whether the modulation of MMP is correlates with a modulation of TIMPs. Human monocytes were cultured in the presence of 6 different HA powders or zymosan. Following 6 h and 18 h after incubation, cells were harvested and TIMP-1 and TIMP-2 mRNAs steady-state levels were determined by RT-PCR analysis. After 6 h of culture, we showed a strong increase of both TIMP-1 and TIMP-2 (Figure 4) mRNA expression in all conditions in regard to the control cells. In contrast, after 18 h of culture no important difference was shown for the expression of TIMP-1 in presence of all HA powders or zymosan. But, we showed a large decrease of TIMP-2 mRNA expression when cells were cultured in presence of needle particles (HA6) or zymosan particles. With any other powders TIMP-2 mRNA expression was slightly increased.

The expression of TIMP-1 and TIMP-2 was not sensitive to the sintering temperature and the size of the particles. Only the shape induced a change in the expression of TIMP-2.

IL-1β production by human monocytes exposed to HA or zymosan particles

To study the HA-mediated influence on IL-1β expression, the most inductor of MMPs, by human monocytes in cultures, cells were cultured in the presence of 6 different HA powders or zymosan particles as positive control. Following 6 h and 18 h after incubation, cells were harvested and IL-1β mRNA steady-state levels were determined by RT-PCR analysis.

As presented in figure 5A, human monocytes expressed constitutively IL-1β mRNAs.

After 6 h of incubation, treatment of cells with all HA particles and zymosan particles lead to an increase of IL-1β mRNA expression (Figure 5A). On the other hand, after 18h of incubation, we showed that only the phagocytable spherical particles (HA1, and HA2), the needle particles (HA6), and zymosan particles induce an increase of IL-1β mRNA expression. In presence of non-phagocytable spherical particles (HA3) a decrease of IL-1β mRNA expression was shown. Using indifferent shape particles (HA4, and HA5) expression of IL-1β mRNA was close to the control one.

To determine whether the modulation of IL-1β mRNA following HA or zymosan treatment is associated with a concomitant protein secretion; conditioned medium collected 6 h and 18 h after activation of cells were analyzed by ELISA (Figure 5B). After 6 h and 18 h of culture, phagocytable spherical (HA1 and HA2) and irregular particles (HA4 and HA5) induced an increase of the production of IL-1β. On the other hand, HA3 particles (not phagocytable) did not changed the production of IL-1β. Only needle particles (HA6) and zymosan particles lead to an important liberation of IL-1β in the cell-free supernatant of monocytes exposed to HA particles and zymosan particles after 6 and 18 hours.

The particles sintering temperature did not modify the production of IL-1β. But the size and the shape of the particles induced changes in the production of the cytokine studied.

Discussion

Hydroxyapatite is widely used in orthopaedic surgery for their biocompatibility and bioactivity. Aseptic loosening remains the primary cause of failure in total arthroplasty, and the process of osteolysis is in part due to the production of particulate debris from the material[2,4]. To assess their effects on MMP-2, MMP-9 production and their inhibitors TIMP-1 and TIMP-2 on human monocytes, we have used 6 different hydroxyaptite particles with various physical characteristics.

Effects of the sintering temperature of HA particles

We have evaluated the effect of the sintering temperature on the production of MMP-2 and MMP-9 using two spherical particles (HA1 and HA2) and two irregular particles (HA4 and HA5) sintered at 600°C or 1180°C with the same size. In both cases of shape, particles sintered at different temperature induced the same expression of MMP-2. But no pro- or active MMP-2 were obtained in the conditioned media (CM) or in the cellular extracts (CE). It has already been demonstrated that the regulation of MMPs is complex, and occurs at both transcriptional and post-transcriptional levels. The complex formation with TIMPs inhibits the activity of MMPs[31]. So, the lack of MMP-2 be due to the large expression of TIMP-2 mRNA at the same time. After 18 hours, the expression of TIMP-2 and MMP-2 was similar to the control one. It means that the expression of MMP-2 and TIMP-2 was correlated.

After 6 and 18 hours, the expression of MMP-9 was the same whatever the sintering temperature. Quantity of pro-MMP-9 in the CM was the same for both groups of particles (spherical or irregular) after 6, 18, and 24 hours. In the CE, both pro- and active MMP-9 were more present using HA1 than HA2 whatever the time. Using irregular particles, we observed no difference in the activity of MMP-9. A high level of TIMP-1 mRNA expression was found in the case of high level of MMP-9 mRNA expression only after 6 hours. There was no

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difference in the expression of TIMP-1 using the two irregular particles. After 18 hours, the expression of TIMP-1 was similar whatever the sintering temperature of spherical or irregular particles. We obtained a similar large production of IL-1β whatever the sintering temperature. So the increase of active MMP-9 with the HA1 can not only be attributed to IL-1β overproduction like Liacini et al.[19] observed in stimulated chondrocytes.

Few authors have studied the effect of the surface area on the MMPs production. Using different material (titanium) and different cells (fibroblast cells), Chou et al.[32] observed variations in the expression of MMP-2 on smooth titanium surface after few hours (1.5 and 3) compared to rough surface. As in the present study, they observed no changes in the quantity of MMP-2 after 16 and 24 hours compared to their control. In their work, they did not study TIMP-2, MMP-9 or TIMP-1.

So, we can conclude that the sintering temperature modify weakly the production of MMPs and TIMPs.

Effects of the size of HA particles

To evaluate the effect of the particles size on MMP-2 and MMP-9 production, we used phagocytable spherical particles (HA2) and bigger spherical particles that were not phagocytable (HA3), both were sintered at 1180°C. Whatever the size of the particles (phagocytable or not), the expression of MMP-2 was similar after 6 and 18 hours. With both particles, there was pro- or active MMP-2 neither in the CM nor in the CE. The quantity of TIMP-2 was the same in both cases. The expression of MMP-9 was also quite the same using both kinds of particles. But the quantity of pro- and active MMP-9 was the higher in the CM or in the CE using not phagocytable particles. The down-regulation of the production of MMP-9 by TIMP-1 was independent of the particle size whatever the time since the

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expressionof TIMP-1 was the same in both cases oh HA particles. MMPs production and IL-1β was not connected : although, there was less IL-1β and more MMP-9 using non-phagocytable particles than using phagocytable particles. Using fibroblasts, Cheung et al.[33] observed that the endocytosis was required to induce synthesis of metalloprotease (collagenase and stromelysin). These apparently opposite results may be explain by a different cell culture method (they used the same amount of powder whatever the size of their particles that is incompatible with the SAR used in the present work) or by of the cells used (fibroblasts or monocytes).

From these data, we can establish that the size of HA particles seems to influence the MMPs production even if it was not correlated to the IL-1β production.

Effects of the shape of HA particles

The effect of the particles shape was studied using three different particles of the same size (1-30 μm) sintered at the same temperature (600°C) that were spherical, irregular or needle shaped (HA1, HA3, and HA6). After 6 and 18 hours, the expression of MMP-2 was quite the same whatever the shape. After 6 hours, the expression of TIMP-2 was the same for all kinds of particles. After 18 hours, the expression of TIMP-2 was the same using spherical and irregular particles but it was very low using needle particles. After 6, 18 or 24 hours, needle particles induced the most important expression of MMP-9 mRNAs and the most important quantity of pro-MMP-9 in the CM. In the CE, whatever the time of cell culture, the quantity of pro-MMP-9 was weak using needle particles. This can be explain by the fact that in the same condition, needle particles induced the most important activity of the active form of MMP-9. The production of IL-1β was also more important using needle particles. Expression of TIMP-1, using zymosan or needle particle was comparable. Using spherical and irregular

particles, there was no difference in the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNAs. In the same way, there was few difference of the activity of pro or active MMP-9 in the CM and CE such as in the production of IL-1β. Using needle particles, there was a correlation between the production of IL-1β and the production of MMP-9 like it has already been observed[34]. It is interesting to note that cells did react on the same way when they are exposed to needle particles or to zymosan particles (an inflammatory particles). Indeed Laquerriere et al[20]. obtained a similar effect on the TNF-α, IL-6 and IL-10 production, using the same needle particles or zymosan particles.

To our knowledge, this work is the first study, that points out a shape effects of the wear debris on the production of MMP-2, MMP-9 and their inhibitors TIMP-2 and TIMP-1.

Conclusions

It is of major importance to understand the effect of biomaterials on cell physiology. We demonstrate that the physical characteristics of the hydroxyapatite have an influence on the production of MMP-2 and MMP-9 and on their inhibitors TIMP-2 and TIMP-1. The sintering temperature that change the surface area did not alter the production of MMP-2 or MMP-9. Surprisingly, non-phagocytable particles increased the production of metalloprotease studied. The parameter that influenced the most the cells were the shape of the particles. Cells exposed to needle particles induced the most important production of MMP-9. These facts has to be taken into account when prosthesis are made.

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Captions

Table 1 : Particles characteristics.

Table 2 : Nucleotides sequence of primers used for PCR and expected sizes of PCR products.

Figure 1 : mRNA expressions of MMP-2 and MMP-9 by human monocytes exposed to HA particles.

Monocytes were treated with six hydroxyapatite and zymosan particles. After 6 and 18 hours, total RNA was isolated and 0.4 μg were reverse transcribed and subjected to PCR to quantify the expression of MMP-2 and MMP-9. The β-actin was the internal RT-PCR control. Results are shown as photography of gels (A) and as semi-quantification of signals of MMP-2 (B) and of MMP-9 (C). We present here results obtained on one donor. We obtained the same kind of results with all of the donors (three).

Figure 2 : Activity gelatinolytic of MMPs in conditionned media of human monocytes exposed to HA particles.

Monocytes were treated with six hydroxyapatite and zymosan particles for 6, 18 and 24 hours. Conditionned media were then harvested and pro-MMPs activity was analysed by gelatin zymography. Results are shown as invert photography of gels (A) and as semi-quantification of signals of the pro-MMP-9 (B). We present here results obtained on the same donor as figure 1. We obtained the same kind of results with all of the donors (three).

Figure 3 : Activity gelatinolytic of MMPs in cellular extracts of human monocytes exposed to HA particles.

Monocytes were treated with six hydroxyapatite and zymosan particles for 6, 18 and 24 hours. Cellular extracts were obtained after lysis as described in the Material and Methods section then pro-MMPs and active MMPs activities were analysed by gelatin zymography. Results are shown as invert photography of gels (A) and as semi-quantification of signals of the pro-MMP-9 (B) and of the active MMP-9 (C). We present here results obtained on the same donor as figure 1. We obtained the same kind of results with all of the donors (three).

Figure 4 : mRNA expressions of TIMP-1 and TIMP-2 by human monocytes exposed to HA particles.

Monocytes were treated with six hydroxyapatite and zymosan particles. After 6 and 18 hours, total RNA was isolated and 0.4 µg were reverse transcribed and subjected to PCR to quantify the expression of TIMP-1 and TIMP-2. The β-actin was the internal RT-PCR control. Results are shown as photography of gels (A) and as semi-quantification of signals of TIMP-1 (B) and of TIMP-2 (C). We present here results obtained on the same donor as figure 1. We obtained the same kind of results with all of the donors (three).

Figure 5 : Synthesis of IL-1β by human monocytes exposed to HA particles.

Monocytes were treated with six hydroxyapatite and zymosan particles. After 6 and 18 hours, total RNA was isolated and 0.4 µg was reverse transcribed and subjected to PCR to quantify the expression of IL-1β. The β-actin was the internal RT-PCR control. Results are shown as photography of gels (Aa) and as semi-quantification of signals of IL-1β (Ab). The production of IL-1β in cell-free supernatant was determined by ELISA 6 and 18 hours following incubation with hydroxyapatite and zymosan particles (B). We present here results obtained

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on the same donor as figure 1. We obtained the same kind of results with all of the donors (three).

Particle shape	Number of the powder	Size range (μm)	Sintering temperature (°C)	Specific surface ($m^2.g^{-1}$)	Crystal size (nm)	Shape factor
spherical	**HA 1**	1-30	600	23.95	190	1.20±0.29
	HA 2	1-30	1180	5.38	350	1.20±0.26
	HA 3	170-300	1180	0.50	350	1.27±0.20
irregular shape	**HA 4**	1-30	600	26.72	180	1.24±0.26
	HA 5	1-30	1180	6.08	350	1.30±0.36
needle	**HA 6**	1-30	600	13.18	300	2.10±0.93

Table 1

mRNA	Sense Primer	Antisense Primer	PCR product, bp
MMP-2	5'-GGCTGGTCAGTGGCTTGGGGTA-3'	5'-AGATCTTCTTCTTCAAGGACCGGTT-3'	225
MMP-9	5'-GCGGAGATTGGGAACCAGCTGTA-3'	5'-GACGCGCCTGTGTACACCCACA-3'	208
TIMP-2	5'-CTCGGCAGTGTGTGGGGTC-3'	5'-CGAGAAACTCCTGCTTGGGG-3'	364
TIMP-1	5'-ACCATGGCCCCCTTTGAGCCCCTG-3'	5'-TCAGGCTATCTGGGACCGCAGGGA-3'	627
IL-1β	5'-TACGAATCTCCGACCACCACTACG-3'	5'-GTACAGGTGCATCGTGCACATAAGC-3'	209
β-actin	5'-TGCTATCCAGGCTGTGCTA-3'	5'-ATGGAGTTGAAGGTAGTTT-3'	443

Table 2

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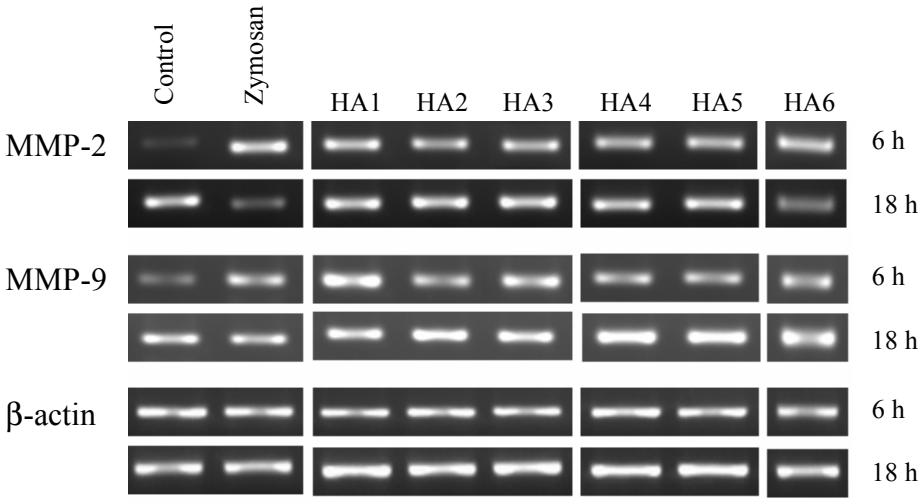
A



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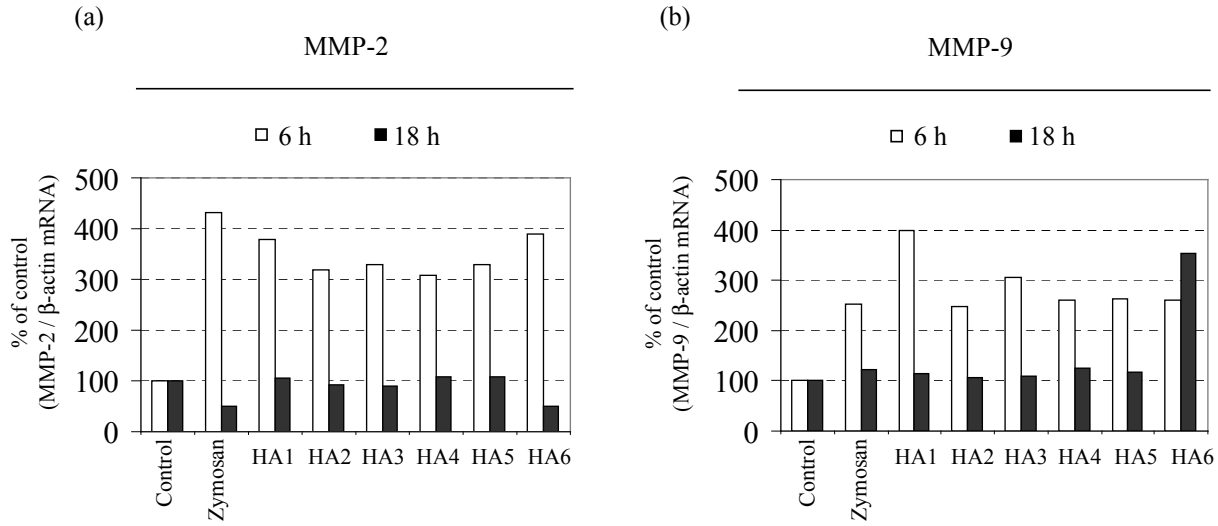

Figure 1

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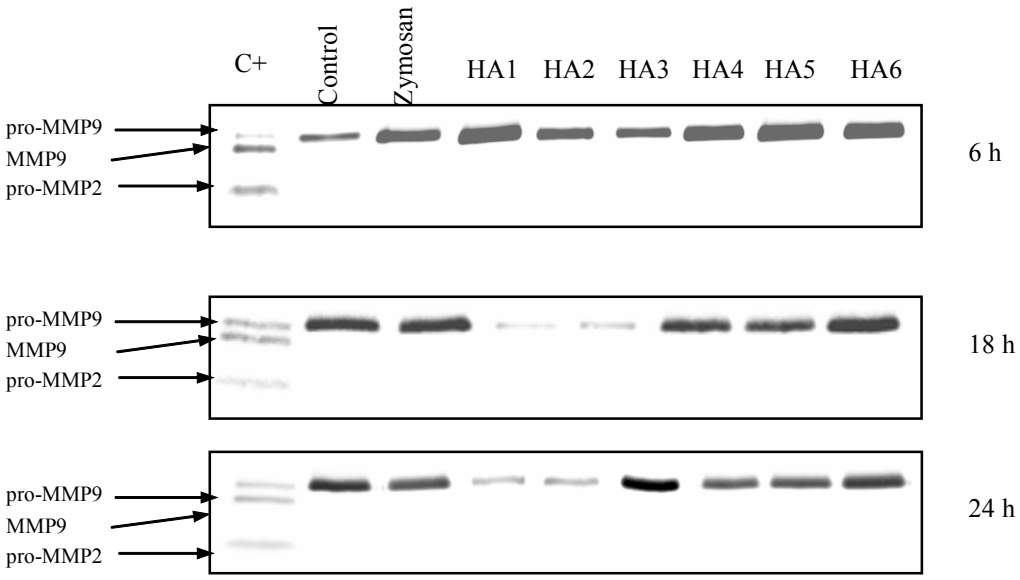

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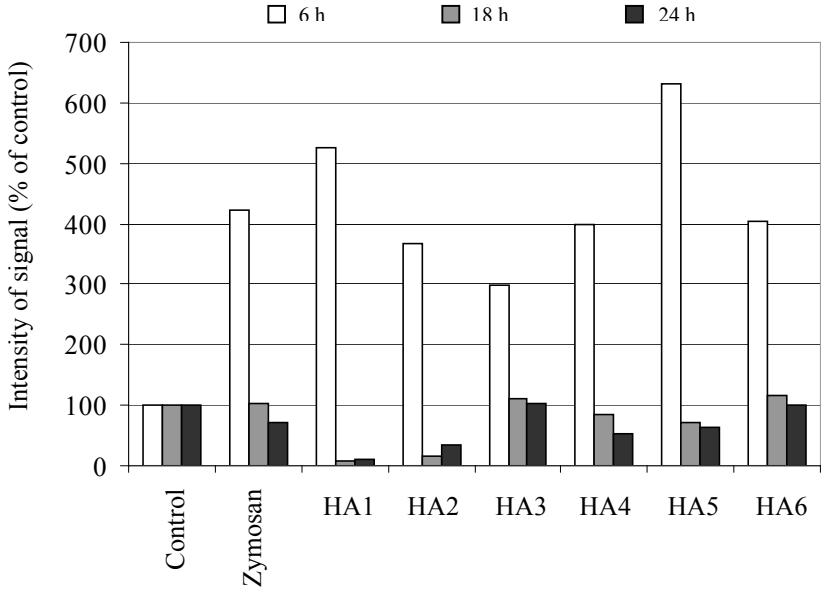

Figure 2

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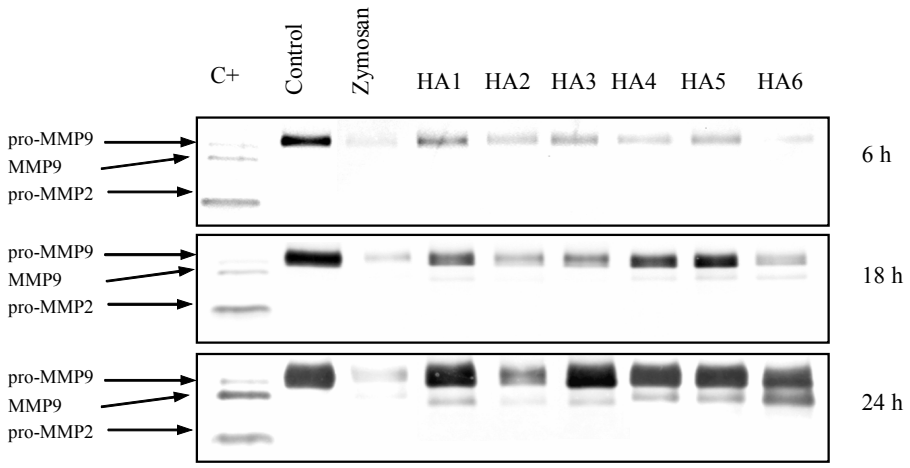
A



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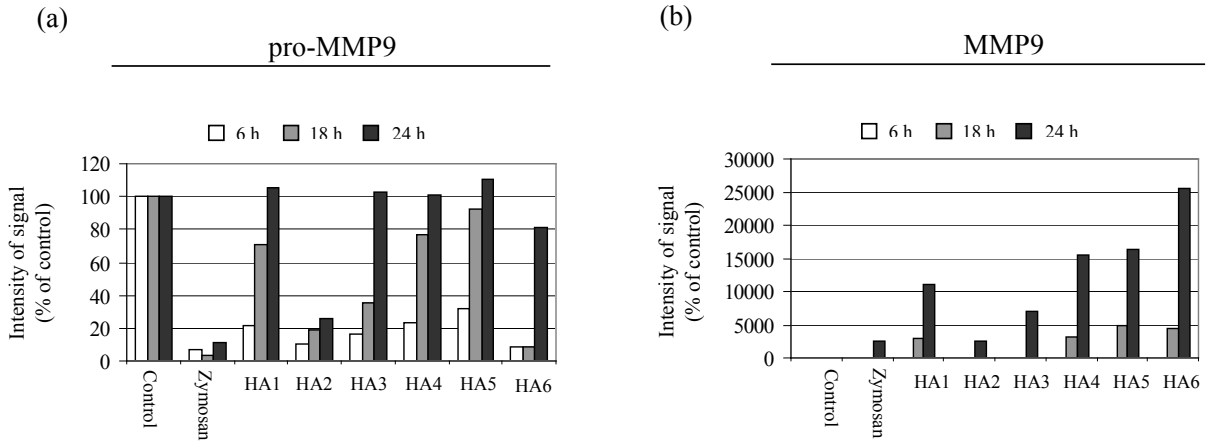
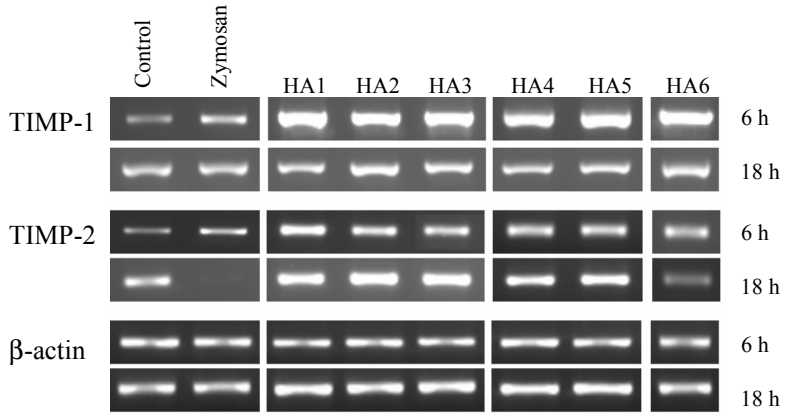


Figure 3

A



B

(a)

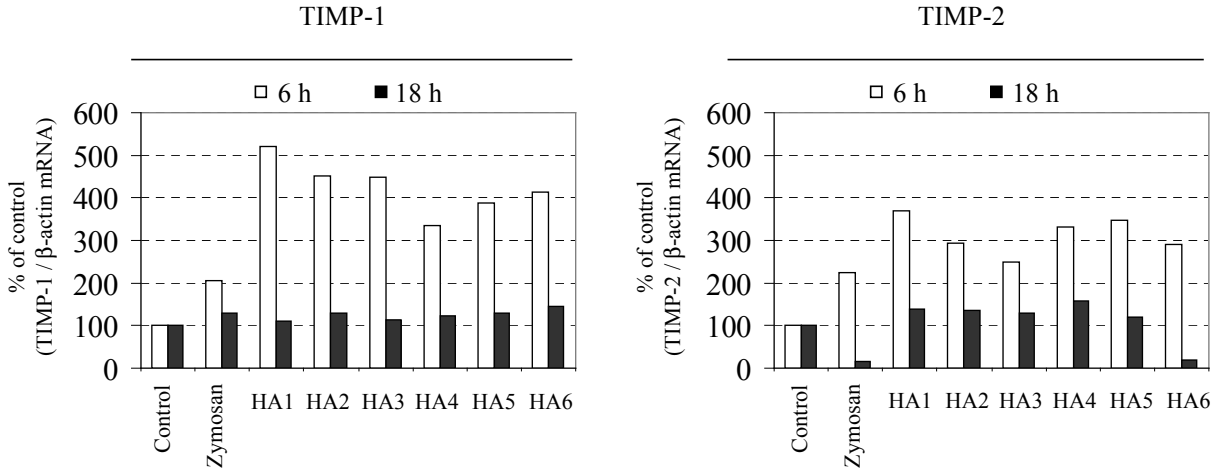
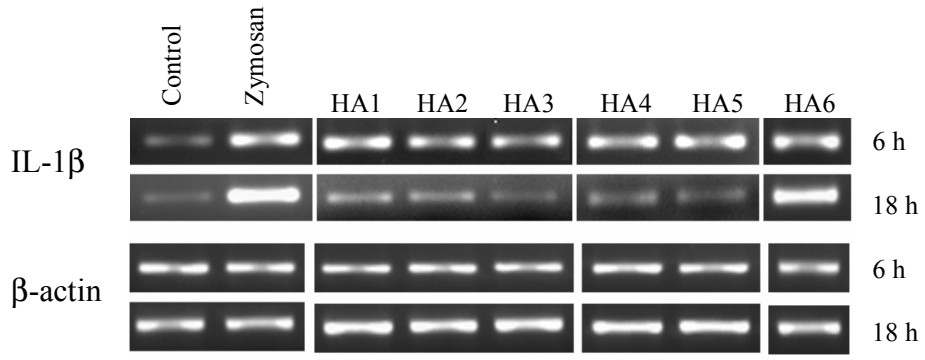


Figure 4



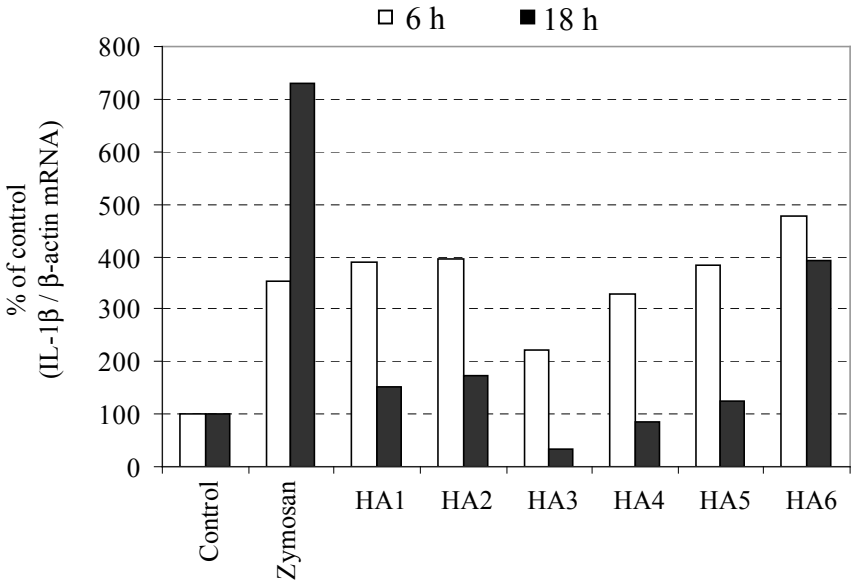


Figure 5