Role of interleukin-1β and tumour necrosis factor-α on hydroxyapatite-induced phagocytosis by murine macrophages (RAW264.7 cells)

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Hydroxyapatite (HA) is used in bone reconstruction in dental and orthopaedic surgery. Characteristic features of HA such as bioactivity, osteoconduction, osteoinduction and the fact that it is similar in composition to bone mineral are factors that favour its use in regeneration of hard tissues.[1] However, when HA is implanted in tissue, monocytes /macrophages are attracted to the implant site.[2] Analysis of tissue from animal models[3] and humans demonstrate the phagocytosis of HA particles.[4]

The most common causes of failure in implants is aseptic loosening, which is mostly triggered by wear particles that activate macrophages to produce cytokines such as interleukin (IL)-1 and IL-6, thereby initiating bone resorption around the implant.[5] It has been shown that both IL-1β and tumour necrosis factor-α (TNFα) are identified in the periprosthetic tissues of patients who undergo revision surgery for total joint replacement,[6] indicating that these cytokines play a crucial role in inflammation and osteolysis. Indeed, implant materials such as titanium and alumina ceramic particles stimulate the production of pro-inflammatory cytokines by murine or human macrophages *in vitro*.[7,8]

Recently, this group showed that murine macrophage phagocytic activity induced by HA is under the regulation of inducible nitric oxide synthase (iNOS).[9] Therefore, this study aims to evaluate the effect of HA-induced phagocytosis on IL-1β and TNFα production and to demonstrate whether or not murine macrophages (RAW264.7 cells) use these cytokines in an autocrine fashion.

Hydroxyapatite powder (3.5–8 μm particle size) was suspended in sterile saline.[10] Latex beads (3 μm; Sigma, St. Louis) were used as a control. The HA particles were stained with crystal violet prior to use in the phagocytic assay.

Hydroxyapatite-induced phagocytosis by RAW264.7 cells was assessed as previously described.[9] Briefly, RAW 264.7 cells, obtained from ATCC, were cultured in Dulbecco's modified Eagle's medium (supplemented with 1% penicillin/streptomycin) and 10% heat-inactivated fetal calf serum in a humidified atmosphere of 5% CO_2. Five million cells were incubated with 1 x 10^5 HA particles or latex beads in culture medium (1 mL) in a sterile tube for 7, 15, 30 and 60 min. At each time point, culture supernatants were harvested and IL-1β and TNFα levels were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. Unstimulated cell culture supernatants were used as a control. All materials used in the cell culture process were obtained from Sigma.

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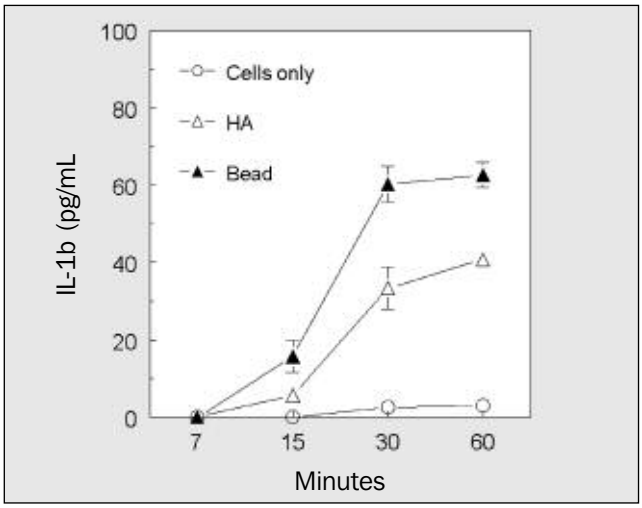

Fig. 1. Effect of HA and latex bead particles on the release of IL-1β by RAW264.7 cells at various incubation times. Untreated cell culture supernatants were used as negative controls. The bars represent standard deviation.

RAW264.7 cells were stimulated with HA or latex beads in the presence of various concentrations of anti-murine IL-1β or TNFα antibody (Sigma). Phagocytic index (PI) was assessed at 60 min. The number of phagocytosed cells per 100 cells was assessed by light microscopy (Leica, Germany). The PI was calculated by counting the number of engulfed particles per macrophage, as described previously.[11] All experiments were carried out in triplicate.

Data analysis included paired *t*-test at different time points and one–way analysis of variance followed by Fisher's least squared difference in the experiments where neutralising IL-1β and TNFα antibodies were used with activated cells (SPSS, Chicago, IL).

IL-1β and TNFα were detected at 30 and 60 min in unstimulated RAW264.7 cell cultures (Figs. 1 and 2). Following activation of the cells, IL-1β concentration was higher in cultures treated with latex beads than with HA ($P<0.05$; Fig. 1). Murine macrophages activated with HA and latex bead particles produced significantly higher levels of IL-1β when compared to unstimulated controls at 15, 30 and 60 min ($P<0.05$). Levels of IL-1β were not detected at 7 min following activation with HA or latex beads. Examining the trend within groups, IL-1β production increased at 7–15 and 15–30 min ($P<0.05$) but did not increase significantly at 30–60 min following activation with HA or latex beads.

TNFα levels in the cultures treated with HA were higher than in those treated with latex beads ($P<0.05$; Fig. 2). Examining the trend within groups, TNFα production showed significant increases at 7–15 ,7–30 and 7–60 min ($P<0.05$); however, the HA-treated group also showed significance at 15–60 min ($P<0.05$) but did not increase significantly at 15–30 and 30–60 min. In the latex bead-treated group, TNFα production did not increase significantly at 15–30, 15–60 or 30–60 min.

Pretreatment of cells with anti-murine IL-1β antibody

humans remains speculative; however, they confirm previous reports that show HA-stimulated human macrophages produce TNFα and IL-1β,[16,17] which suggests that HA-stimulated RAW264.7 cells are a good model of HA-induced human macrophage responses. Furthermore, when used as an implant in humans, HA activates macrophages,[24] and analysis of periprosthetic tissues from patients with implants has identified cytokines such as TNFα and IL-1β.[6]

Thus, it seems plausible that macrophages are activated immediately after implantation with HA and then subsequently produce TNFα and IL-1β. This may enhance macrophage infiltration and function and induce pro-inflammatory responses to surrounding implanted materials, as is seen in other pathophysiological disease processes.[25]

In summary, the results of the present study show that HA stimulates murine macrophages (RAW264.7 cells) to produce IL-1β and TNFα; however, addition of neutralising antibodies to IL-1β or TNFα result in reduction of particle-induced phagocytosis. This suggests that HA-induced phagocytic activity by murine macrophages is dependent on the presence of IL-1β and TNFα.

This research was supported by an e-IRPA category EA grant (No. 06-02-05-00029 EAR) from the Malaysian Government. The hydroxyapatite was kindly prepared by staff of the School of Material and Mineral Resources Engineering, Universiti Sains Malaysia, Malaysia.

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