Development of an *In Vitro* Model on Cellular Adhesion on Granular Natural Bone Mineral Under Dynamic Seeding Conditions—A Pilot Study

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Abstract: Adhesion of osteogenic cells on biomaterials can be studied with static *in vitro* models, whereas models representing dynamic seeding conditions are rare. Herein, we present an *in vitro* model to study cell adhesion on granular biomaterials under dynamic seeding conditions. Radiolabeled osteogenic MC3T3-E1 cells were allowed to adhere to granules of natural bovine bone mineral (NBM) under constant rotation. Adhesion of MC3T3-E1 cells was determined by liquid scintillation counting, and cell morphology was visualized by scanning electron microscopy. Cell viability was determined by MTT assay under static and dynamic conditions, at room and body temperature, and in the presence or absence of serum. We show here that MC3T3-E1 cells rapidly adhere to NBM, reaching a peak 3 h after seeding. Attached cells display characteristic signs of spreading. Five to ten percent of total radioactivity remained on NBM after the removal of nonadherent cells. Viability is maintained at room temperature and under rotation for upto 3 h. This data suggests that the dynamic *in vitro* model presented here provides a tool to study cell adhesion on granular biomaterials. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 91B: 766–771, 2009

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INTRODUCTION

Bone substitutes are biomaterials[1–3] that provide a scaffold for host-derived osteogenic cells but lack the inherent osteogenic potential of autografts.[1–3] To overcome these limitations, bone substitutes can be supplemented with osteogenic cells,[4,5] which requires cell adhesion.[6–8] *In vitro* models to study cell adhesion are mainly performed under static conditions.[9–11] However, a homogenous distribution of cells within granular biomaterials can only be achieved by a dynamic approach. Here, we introduce an *in vitro* model that allows us to examine the adhesion efficacy of osteogenic cells to granular biomaterials under dynamic conditions.

The *in vitro* model presented here was developed with a clinically approved biomaterial and a widely used osteogenic cell line. Bio-Oss® is a granular deproteinized NBM, which is frequently applied in oral and maxillofacial surgery.[12–15] Adhesion of osteogenic cells to NBM blocks[10,16,17] or particles[18,19] was studied under static conditions with MC3T3-E1 cells, a nontransformed osteogenic cell line derived from murine calvaria.[20] MC3T3-E1 are considered as osteogenic cells, as they can express characteristic differentiation markers and can cause bone formation when transplanted to ectopic sites.[21,22] NBM and MC3T3-E1 can, therefore, be considered as prototypes of biomaterials and osteogenic cells, respectively.

In this *in vitro* model, dynamic seeding conditions were achieved by rotation of reaction tubes containing radiolabeled MC3T3-E1 cells and NBM particles on a platform, followed by the quantification of the osteogenic cells adhering to the granular biomaterial via liquid scintillation counting.

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MATERIAL AND METHODS

Natural Bone Mineral

Bio-Oss® (NBM; Geistlich Pharma, Wolhusen, Switzerland) is a sterilized, highly porous natural xenograft recovered from bovine bone. The organic components are removed by treatment with alkaline solution and subsequent sintering at 300°C.[23] The porosity of Bio-Oss® is 70–75%. Bio-Oss® with a granular size ranging from 0.25 to 1 mm was used. Crystal and morphological structures are similar to human trabecular bone. Bio-Oss® provides an interconnecting pore system that enlarges the inner surface and allows the immigration of osteogenic cells and the process of angiogenesis to occur.[24]

Basic Experimental Setting

MC3T3-E1 cells were kindly provided by Dr. F. Varga (Ludwig Boltzmann Institute of Osteology, Vienna, Austria). Cells were cultured in αMEM (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; PAA, Linz, Austria), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin (Gibco) in a humidified atmosphere at 37°C. For the experiments, MC3T3-E1 cells were seeded into T-125 flasks with a density of 5×10^4 cells/cm^2 in the presence of 2 μCi/mL ^{3}[H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK). The following day, radiolabeled cells were harvested, resuspended in serum-free medium, and transferred into reaction tubes with the capacity of 2 mL (Eppendorf, Hamburg, Germany) previously filled with aliquots of 50 ± 5 mg NBM. The tubes were placed onto a platform (Rotator Stuart, VWR International, Vienna, Austria) turning at five revolutions per minute. If not otherwise indicated, the culture medium was withdrawn and NBM particles were washed with phosphate-buffered saline (PBS) after 3 h of incubation at room temperature. NBM particles were allowed to dry completely before MC3T3-E1 cells were lysed with 300 μL of a 100 μM NaOH. Radioactivity of the lysates was measured via liquid scintillation counting (TopCount, Packard, Meriden, CT). Data are given as counts per minute. Experiments were performed in triplicates and were repeated at least twice.

Determination of Dosing, Timing, and Pretreatment

We first determined the optimum concentration of cells and the optimum time period suitable to perform the dynamic seeding process in an economic and efficient way. MC3T3-E1 cell–doses ranging from 1×10^5 to 1×10^7 cells/mL were added to the biomaterial resulting in a cell to NBM-ratio of 2×10^3 to 2×10^5 cells/mg NBM in 1 mL serum-free medium. This suspension underwent constant rotation for 3 h. To determine the time of maximum cell adhesion, 1×10^6 MC3T3-E1 cells were seeded on 50 mg NBM in 1 mL serum-free medium and underwent constant rotation for 1, 3, and, 6 h. These experiments were repeated using primary rat bone marrow stromal cells and the osteogenic cell lines ST2 and hFOB (data not shown). To determine the influence of pretreatment of NBM with FCS or PBS on cell adhesion, NBM particles were incubated with 100 μL FCS or PBS for 1 h at room temperature before being washed with PBS. Radiolabeled MC3T3-E1 cells were then added to the pretreated NBM and were processed according to the basic experimental setting.

Determination of Absolute and Relative Numbers of Adherent Cells

Suspensions of NBM and radiolabeled MC3T3-E1 cells at doses ranging from 3×10^4 to 1×10^6 cells/50 mg NBM were subjected to an incubation period of 3 h. To assess the percentage of adherent cells, NBM were washed or remained unwashed before liquid scintillation counting was performed. Based on these data, the absolute number of cells adherent to NBM and the relative number of cells per number of seeded-cells adherent to NBM were calculated based on a calibration curve.

Scanning Electron Microscopy

Morphological features of MC3T3-E1 cells were determined after 3 h of rotation using scanning electron microscopy (Quanta 3D, FEI Company, Hillsboro, OR). Samples were fixed in 2.5% glutaraldehyde (Sigma) for 30 min. After rinsing with PBS, cells were dehydrated in ascending concentrations of ethanol and allowed to dry. Cell morphology on the surface of the NBM was visualized in the low vacuum modus with a large field detector.

Cell Viability Assay

To evaluate the impact of the culture conditions on cell viability, we investigated the influence of temperature (room temperature *vs.* 37°C), the presence of serum (serum *vs.* serum-free conditions), and the impact of rotation (dynamic *vs.* static conditions) on MTT conversion by the cells. MTT solution (150 μL) (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide; 10 mg/mL; Sigma) were added to the tubes during the 3-h incubation period. The substrate solution was removed and formazan crystals were solubilized with 100 μL dimethyl sulfoxide. Optical density of the solution was measured at 570 nm. Data were expressed as optical density.

Statistical Analysis

Data were analyzed by ANOVA with post-hoc Bonferroni testing. The results represent two independent experiments performed in triplicates for each data point. Significance was assigned at the $p < 0.05$ level.

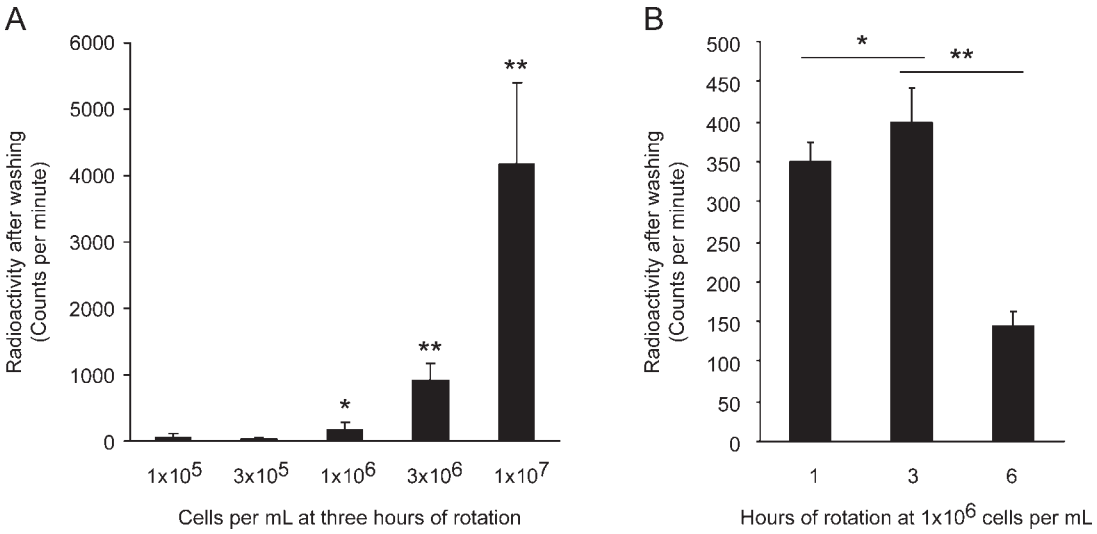

Figure 1. Cell adhesion on NBM under rotation—dosing and timing. NBM together with radiolabeled MC3T3-E1 cells were placed on a rotating platform at different cell concentrations (A) and after different incubation periods (B). The NBM particles were vigorously washed and radioactivity of the adherent cells was determined. Data are given in counts per minute. Experiments were performed in triplicates and represent two independent experiments. Bars represent the mean + standard deviation. $*p < 0.05$, $**p < 0.01$.

RESULTS

The Basic Experimental Setting

To establish the basic experimental setting, radioactivity remaining on the biomaterial at different cell-doses and after different time periods was determined. Dose-response experiments showed that radioactivity remaining on the NBM after washing was higher than background activity at the least significant concentration of 1×10^6 cells per mL and increased at higher concentrations [Figure 1(A)]. Time-response experiments revealed that radioactivity of adherent MC3T3-E1 cells reached a maximum after 3 h and decreased with longer incubation periods [Figure 1(B)].

Evaluation of the Absolute and Relative Numbers of Adherent Cells

Radioactivity corresponding to the total cell number [Figure 2(A)] and radioactivity of the adhering cells [Figure 2(B)] were measured. The number of counts per minute showed a linear rise with increasing numbers of seeded-cells. Similarly, the number of cells remaining attached to NBM after washing increased with the number of seeded-cells. The number of adherent cells [Figure 2(C)] and the percentage of cells remaining on the NBM after washing [Figure 2(D)] was calculated based on a calibration curve. Even though the absolute number of adherent cells increased with the total number of initially seeded-cells, the amount of adherent cells was rather low. The ratio of adherent to seeded-cells decreased with rising doses of seeded-cells. At 3×10^5/mL, ~30% of seeded-cells adhered on the biomaterial, when the highest dose of 1 million cells was seeded, the percentage of adherent cells remaining on the NBM fell below 10% [Figure 2(D)].

Scanning Electron Microscopy and Pretreatment of NBM

The low total amount of adherent cells on NBM is reflected by the occasional appearance of attached cells in the SEM showing characteristic signs of spreading (Figure 3). The relative amount of adherent cells was comparable between MC3T3-E1, bone marrow stromal cells, hFOB, and ST2 (data not shown). We further tested whether or not pretreatment of the biomaterial can change the adhesion properties of MC3T3-E1 cells. The data show that incubation of NBM with PBS-reduced cell adhesion, an effect that was even more pronounced in the presence of FCS (Figure 4).

Cell Viability Assay

To show that osteogenic cells are viable under the basic experimental conditions, we evaluated the influence of temperature and the presence of serum under static and dynamic conditions. Data illustrated in Figure 5 show that cell viability was stable under the basic experimental conditions, but was significantly lower at room temperature when compared with 37°C. The impact of serum on cell viability was neglectable, independent of the temperature. However, rotation at room temperature caused a significant decrease of cell viability when compared with static conditions. Together the data show that cell viability is slightly decreased but still present under the basic experimental conditions, when compared with the physiological conditions of body temperature.

DISCUSSION

Clinical evidence indicates that the adhesion efficacy of osteogenic cells to bone substitutes is a critical determinant in biomaterial research.[25,26] *In vitro* models are valuable

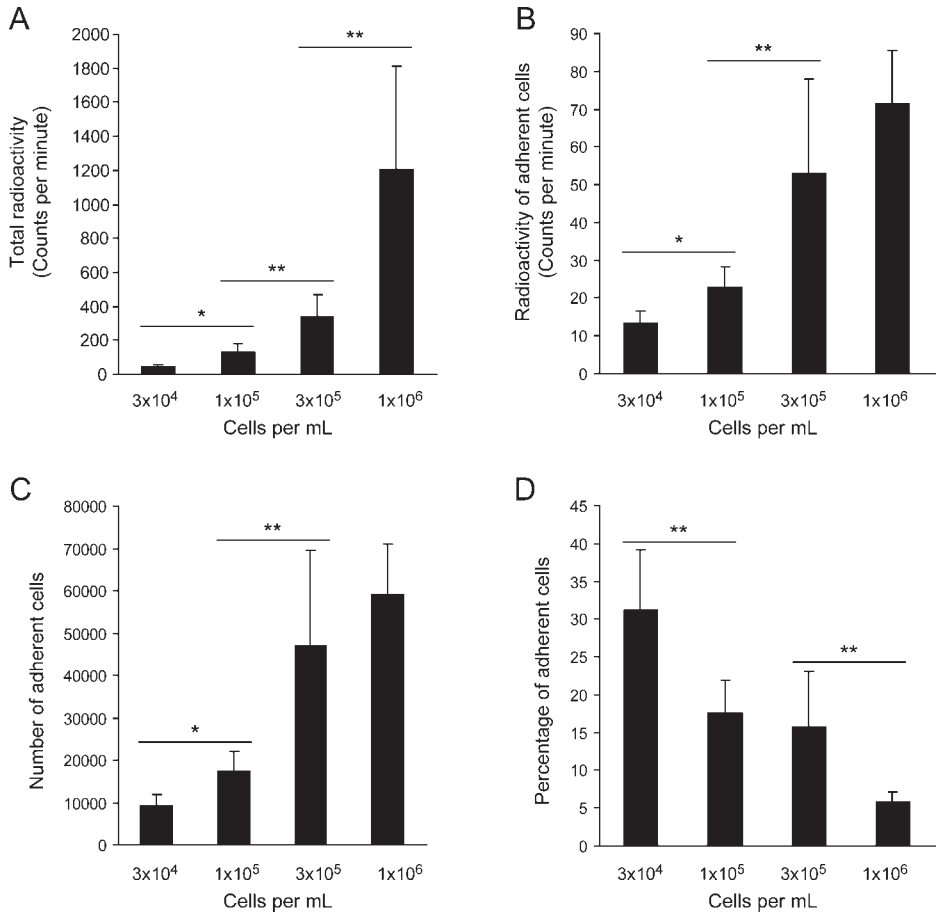

Figure 2. Cell adhesion on NBM under rotation—absolute and relative numbers of adherent cells. NBM together with radiolabeled MC3T3-E1 cells were placed on a rotating platform for 3 h. Radioactivity corresponding to the total cell number {Figure 2(A)} and to the adhering cells {Figure 2(B)} was determined. The number of adherent cells {Figure 2(C)} and the percentage of cells remaining on the NBM after washing {Figure 2(D)} were calculated. Experiments were performed in triplicates and represent two independent experiments. Bars represent the mean + standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$.

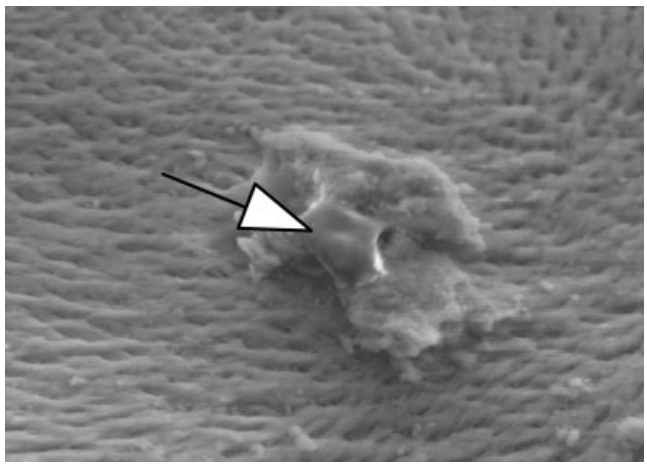

Figure 3. Scanning electron microscopy of adherent cells to the NBM. NBM together with one million MC3T3-E1 cells were placed on a rotating platform for 3 h. NBM particles were washed, fixed with glutaraldehyde, and dehydrated with alcohol series. Samples were subjected to scanning electron microscopy. The white arrow indicates a MC3T3-E1 cell on the surface of a NBM particle. Magnification ×4000.

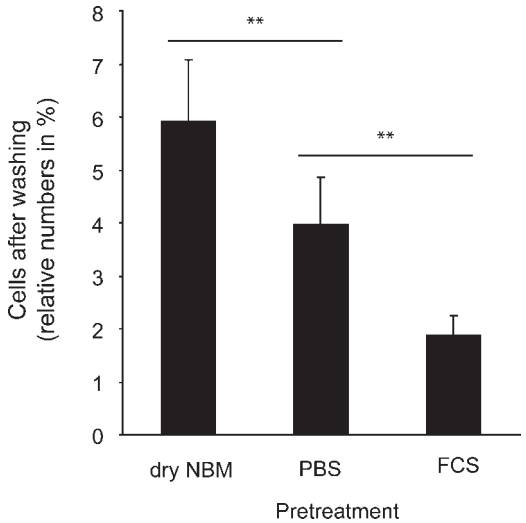

Figure 4. Pretreatment of NBM. To determine the possible effect of pretreatment on cell adhesion, NBM particles were incubated with 100 μL FCS and PBS for 1 h at room temperature following washing with PBS. Radiolabeled MC3T3-E1 cells were added to the tubes and processed according to the basic experimental setting. Bars represent the mean + standard deviation. $^{**}p < 0.01$

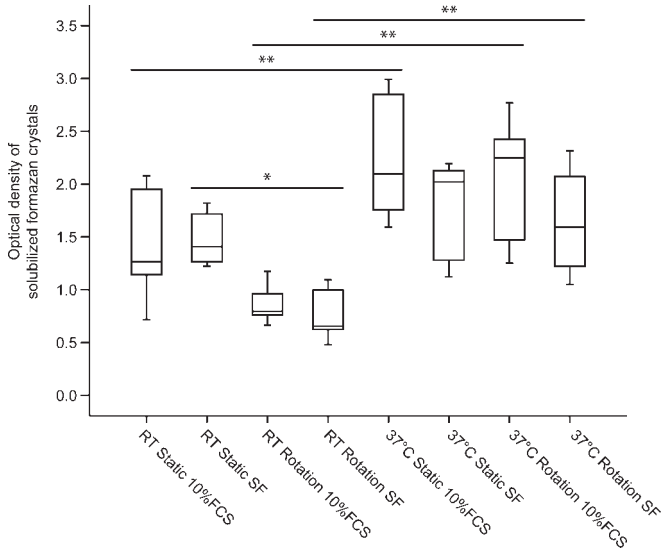

Figure 5. Cell viability assay. NBM was incubated with MC3T3-E1 cells in the presence of MTT under the indicated conditions: (i) temperature (room temperature *vs.* 37°C), (ii) the presence of serum (medium containing 10% fetal calf serum *vs.* serum-free conditions), and (iii) the impact of rotation (dynamic *vs.* static conditions). Data represent that optical density of the solubilized formazan crystals. Box blot represents median, upper, and lower quartiles, and the minimum and maximum data values. $^{*}p < 0.05$, $^{**}p < 0.01$.

tools to optimize the adhesion properties of biomaterials for osteogenic cells.[27] Cell adhesion has been tested under static conditions.[10,16–20] The interaction of osteogenic cells and biomaterials may, however, be different when investigated under dynamic conditions, which are required to achieve a homogenous distribution of cells within granular biomaterials. Aim of this work was to develop an *in vitro* model to study interactions of osteogenic cells with granular natural bone mineral under dynamic conditions.

To reach sufficient sensitivity for liquid scintillation counting, it was necessary to perform the experiments with one million of cells per mL, even though in this setting the percentage of adhering cells is low. The high amount of ^{3}[H]thymidine, which is required to label the cells, is a limitation of the present model. A nonradioactive detection system or a thymidine-free medium to increase the incorporation efficacy of ^{3}[H]thymidine may be administered to overcome these limitations. Cells could also be cultivated with ^{3}[H]thymidine for a time period exceeding 24 h to increase the level of absolute radioactivity, that is, incorporated during cell division.

Our data show that radioactivity remaining on the NBM reaches a maximum between 1 and 3 h and declines thereafter. It is possible that rotation causes mechanical damage of the cells, an effect that becomes even stronger with extended incubation periods. Mechanical cell damage may explain the loss of adhering cells at the 6-h time point. However, the theory that cells are sheared off the NBM particles was not proven in this study. The amount of adhering cells relative to seeded-cells decreased dramatically with increasing doses of seeded-cells. Cell affinity to NBM seems to be weak and can possibly reach a plateau that inhibits a dense colonization of the biomaterial. It is likely to be influenced by time and temperature, however, as a limit of the model the plateau was not reached in this approach.

Serum-free conditions were chosen for the model to rule out possible effects of serum components. The results of this study indicate that serum components are not necessary to maintain cell viability over the short incubation period. These findings also suggest that basal cell viability is maintained under room temperature and under serum-free conditions. In addition, scanning electron microscopy showed that MC3T3-E1 cells display the morphology of spreading cells, another hallmark of cell viability. It is thus possible to perform this dynamic cell seeding experiment at room temperature.

Surface modifications of biomaterials by preincubation with extracellular matrix proteins can influence the adhesion efficacy, as observed with static *in vitro* cultures.[27–29] Data presented here show that pretreatment of NBM with PBS reduced the cellular adhesion, an effect that is even more pronounced when using FCS. These findings were rather unexpected knowing that cell adhesion is usually enhanced in the presence of serum. Still, we have no explanation for these observations, that may be a characteristic phenomenon of the dynamic model described here.

In this *in vitro* system, the absolute and relative numbers of MC3T3-E1 cells adhering to NBM can be calculated based on a calibration curve. Overall, only 5–10% of the originally seeded-cells remained on the NBM after washing, indicating that the rotation model provides highly stringent conditions to determine cell adhesion on biomaterials. The efficacy of cell adhesion can be improved by altering the experimental conditions, particularly the temperature. The influence of the number of revolutions per minute on cell viability remains to be determined. The rotation model presented here can be considered an innovative *in vitro* system and thus offers a complementary method to the static *in vitro* models of cell adhesion.

Even though this study can be considered preliminary, it clearly supports the conclusion that measuring adhesion efficacy under dynamic conditions is possible. In further studies, our method should be adapted to study the adhesion of different cell types to other biomaterials, not restricted to the field of bone regeneration. For example, adhesion of labeled keratinocytes to dermal grafts or the adhesion of endothelial cells to artificial vessels can be studied and may be optimized in a dynamic mode. Another application would be the small scale studying of cell adhesion to carriers in bioreactors, which are may be the best representatives of a dynamic adhesion system that has gained considerable relevance. Optimization of the present protocol also involves variations in the rotation protocol such as longer incubation periods and investigation of the influence of rotation speed as well as different models of movement that may reflect better the irregularity of a bio-

logical state. It should also be stated that the labeling with radioactivity is only one option as there is the possibility of fluorescence tags. Thus, one has to critically consider the method which is suitable to answer a given question. The study presented here should not be considered as a strict protocol—it could be regarded as a primer for establishing an individual system to study cell adhesion under dynamic conditions.

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