Manipulation of Human Primary Endothelial Cell and Osteoblast Coculture Ratios to Augment Vasculogenesis and Mineralization

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Abstract: Tissue-engineering scaffolds are often seeded with a single type of cell, but there has been more focus on cocultures to improve angiogenesis and bone formation for craniofacial applications. Investigation of bone-derived osteoblasts (OBs) is important because of the use of bone grafts and migration of OBs from native bone into constructs in vivo and therefore, their contribution to bone formation in vivo. The limitation of primary OBs has been their inability to mineralize without osteogenic factors in vitro. Through coculture of OBs and endothelial cells (ECs) and manipulation of the coculture ratio, mineralization can be achieved without osteogenic media or additional growth factors, thus enhancing their utility for tissue-engineering applications. An optimal ratio of EC/OB for vasculogenesis and mineralization has not been determined for human primary cells. Human umbilical vein ECs were cultured with normal human primary OBs in different EC/OB ratios, namely, 10:1, 5:1, 1:1, 1:5, and 1:10 with EC and OB monocultures as controls. The number of vasculogenic networks in a collagen matrix was highest in ratios of 5:1 and 1:1. ECs lined up and formed capillary-like networks by day 10, which was not seen in the other groups. On polystyrene, cells were cocultured with ECs and OBs in direct contact (direct coculture) or separated by a transwell membrane (indirect coculture). At day 21, Alizarin Red staining showed mineralization on the 1:5 and 1:10 direct coculture ratios, with 1:5 having more mineralization nodules present than 1:10. No mineralization was seen in other direct coculture ratios or in any of the indirect coculture ratios. Alkaline phosphatase secretion was highest in the 1:5 direct coculture group. Vascular endothelial growth factor secretion from OBs was present in the 1:5 and 1:10 direct coculture ratios at all time points and inhibited after day 1 in other coculture groups. To improve vasculogenesis, cocultures of primary human ECs and OBs in ratios of 5:1 should be used, but to improve bone formation, the 1:5 direct coculture ratio results in most mineralization.

Key Words: coculture, endothelial cells, osteoblast, human primary cells, bone regeneration, vasculogenesis

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There are many types of cells involved with the formation, repair, and remodeling of bone but bone tissue engineering scaffolds have usually been seeded with a single type of cell.[1] There has been more focus on cocultures, the culture of 2 or more types of cells, in in vitro and in vivo studies to more closely model the natural regeneration of bone and gain more insight into what cell cell interactions can improve on the limited success of bone tissue engineering thus far. The gold standard for regeneration of bone defects remains autologous bone grafts which are limited by donor site morbidity, availability, and decreased efficacy in the elderly.[2,3] Harvests from bilateral iliac crests have enough tissue to cover 8 cm per person.[4] Fully exploring the potential of bone derived primary osteoblasts (OBs) may lead to strategies to extend and expand the use of bone grafts for craniofacial applications.

Endothelial cells (ECs) and OBs have multiple interactions during bone formation. Early coculture studies showed that the ECs accelerate bone formation through angiogenesis and support that there is a synergistic relationship between the 2 cell types.[5–9] Osteoblasts produce angiogenic factors such as vascular endothelial growth factor (VEGF) and matrix components which in turn stimulate ECs to produce osteogenic factors important in the differentiation of ECs into vessel components.[8,10–12] Endothelial cells and formed tubules have also showed prolonged survival when in coculture.[13,14]

Coculture studies of EC and OB have been often been done in 1:1 cell ratios in which the number of ECs is equal to the number of OBs[8,15,16] but the full potential of the cocultures is not recognized without using the correct cell ratios. In a study which looked at angiogenesis in cocultures ratios of primary cells, the cultures with human dermal microvascular EC with human primary OB (EC/OB) in ratios of 1:1 or less had almost no ECs remaining at 1 week, whereas ratios of 5:1 and 10:1 EC/OB showed presence of both types of cells after 1 week.[13]

The effect of cocultured cells in different ratios has been systematically investigated by Ma et al using human umbilical ECs and human marrow stromal cells. Because the marrow cells in coculture require osteogenic media to induce mineralization, these data cannot be extrapolated to bone derived OBs which do not require the media with osteogenic supplements to mineralize while in coculture.[17] The optimal coculture ratios that should be used to increase vascularization or osteogenesis from these types of OBs still have not been determined. Investigation of bone derived OBs is important because of the use of bone grafts and migration of OBs from native bone into constructs in vivo and therefore, their contribution to bone formation in vivo. Limitations of primary OBs have been their inability to mineralize without osteogenic factors in vitro. Achieving mineralization of these cells without the need for the addition of factors such as bone morphogenic protein would enhance their utility for tissue engineering applications.

It is hypothesized that by manipulating the cell ratio of EC to OB in coculture, vasculogenesis and mineralization can be augmented. The ratios investigated in this study (10:1, 5:1, 1:1, 1:5, 1:10) were chosen through review of current EC and OB literature. The purpose of this study was to determine optimal coculture ratios of human primary ECs and OBs to enhance mineralization and vasculogenesis. In addition, the effects of physical contact between ECs and OBs on mineralization and secretion of alkaline phosphatase and VEGF in coculture were examined.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein ECs (Invitrogen, Carlsbad, CA) were cultured in EC media (Lifeline, Walkersville, MD) and used after

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passage 2. Normal human primary OBs (Lonza, Allendale, NJ) were cultured in OB media consisting of ascorbic acid and antibiotics (Lonza) and the cells were used after passage 2.

Cocultures

Coculture experiments were performed in different ratios, 10:1, 5:1, 1:1, 1:5, and 1:10, of ECs to OBs denoted EC/OB. Endothelial cell and OB monocultures were used as controls. All cocultures were cultured in media consisting of 2% fetal bovine serum, 15 ng/mL in sulin growth factor 1, 5 ng/mL endothelial growth factor, 5 ng/mL basic fibroblast growth factor, 50 μg/mL ascorbic acid, 1 μg/mL hy drocortisone, 0.75 U/mL heparin, 10 mM glutamine, and antibiotics in MCDB 131. No VEGF was present in these media and both EC and OB monocultures were able to proliferate in these media.

Direct and Indirect Cocultures on Polystyrene

Polystyrene tissue culture treated 24 well plates (Corning, Edison, NJ) were used to examine mineralization in direct and indi rect cocultures. In direct cocultures, the cells were in physical contact with each other. In indirect cocultures, the cells were separated by transwell membranes with 0.4 μm pores (Corning) so growth factors from the cells could interact with the other cell type without physical cell contact. The direct coculture was performed by mixing the cells in the desired ratios before seeding them on the substrates. For the in direct studies, the OBs were seeded on the well plate surface and ECs seeded on the membrane. The culture medium was changed every other day and 24 hours before each supernatant collection. A 700 μL of me dia was used for each medium change. For the indirect cultures, 600 μL was placed into the well and 100 μL into the membrane insert. At each time point, phase contrast microscopy (Nikon, Melville, NY) was used to image the OBs on each well at 10× (n 4 constructs).

Alizarin Red S Staining

Staining using a 2% vol/vol Alizarin Red S solution at pH 4.1 was performed after 21 days of culture to detect calcium from min eralization. After staining, the monolayer was examined under phase contrast using an inverted microscope at 10× (Nikon). Areas with bright red staining were considered to have calcium present (n 4 samples).

VEGF Enzyme-Linked Immunosorbent Analysis

At days 1, 3, 7, 12, 14, 18, and 21, the supernatant from each well of the polystyrene cultures was collected and frozen at −80°C. The culture medium was changed 24 hours before each supernatant collection. After all samples were collected, enzyme linked immuno sorbent analysis was performed to determine protein concentration of VEGF using commercially prepared plates (R&D Systems, Wiesbaden, Germany) per manufacturer's protocol. Results are expressed as pico gram per milliliter (n 3 samples/time point).

Alkaline Phosphatase Assay

At days 1, 3, 7, 12, 14, 18, and 21, the supernatant from each well was collected and frozen at −80°C. The culture medium was changed 24 hours before each supernatant collection. The AnaSpec Sensolyte FDP Alkaline Phosphatase Assay Kit (Fremont, CA) was used to measure the concentration of alkaline phosphatase in the su pernatant per manufacturer's protocol. Results are expressed as micro gram per milliliter (n 3 samples/time point).

Collagen Angiogenesis Assay

Collagen matrix was used to evaluate the ability of the co cultures to form vasculogenic networks in an environment that sup ports vasculogenesis, but does not contain provasculogenic factors.[15] A 30 μL of ECM matrix (Cayman Chemical, Denver, CO) was

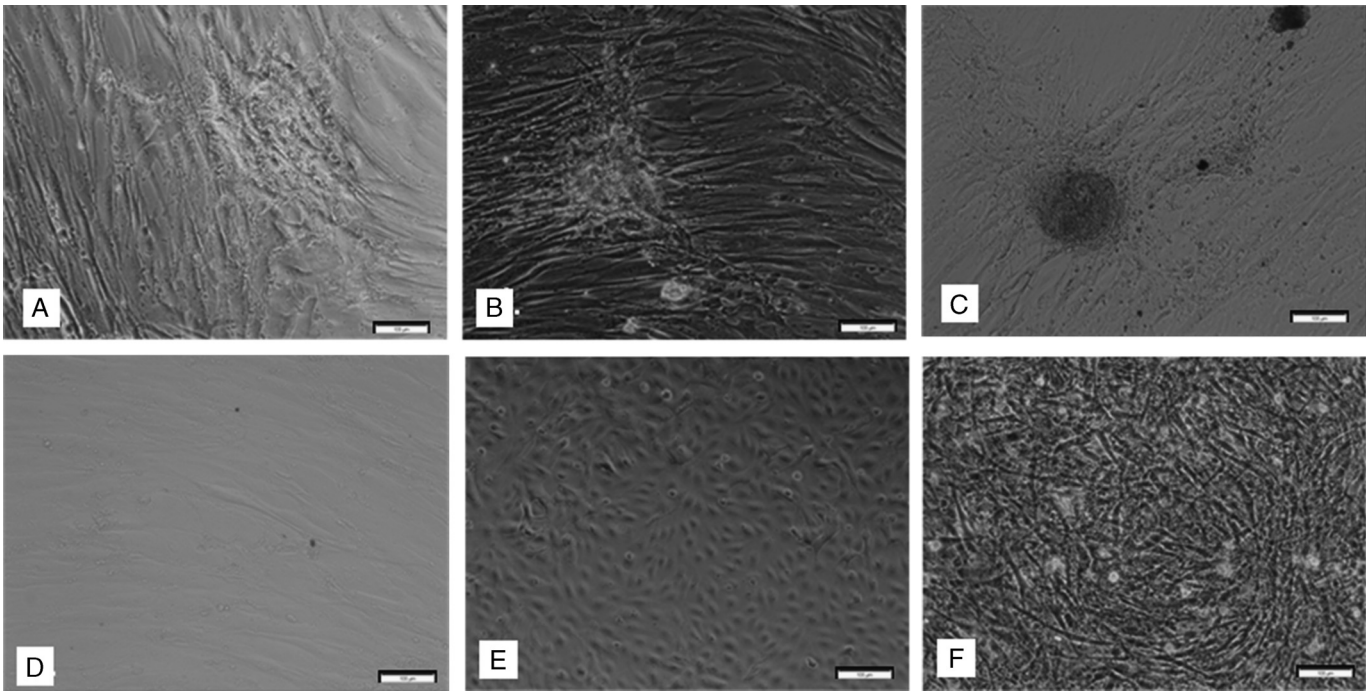

FIGURE 1. Representative images of polystyrene cocultures in direct contact: (A) separation of ECs and OBs in 1:10 coculture on day 7, (B) nodule formations on day 10 of 1:5 coculture, (D) nodule formation at day 21 in 1:5 coculture, (C) OB monoculture on day 21, (E) EC monocultures at day 14 with apoptotic cells, and (F) 10:1 coculture on day 14 with ECs and OBs growing forming a mesh-like network.

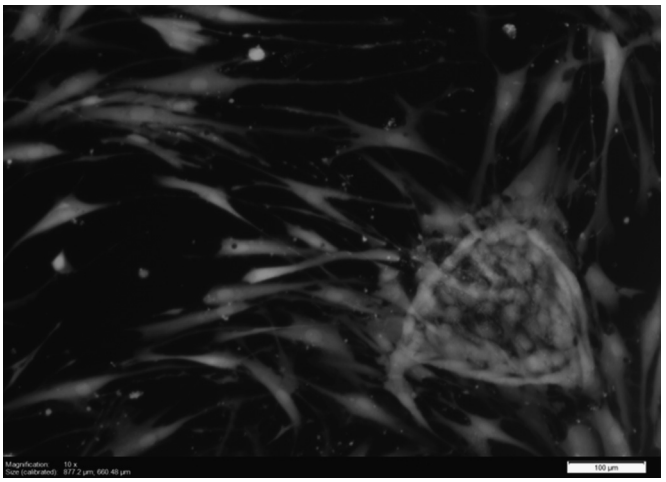

FIGURE 2. Day 7 collagen assay. Circular endothelial formations are seen in the 1:5 coculture.

placed in each well of a 96 well plate and allowed to solidify at 37°C for 1 hour. Cocultures in the following coculture ratios (EC/OB): EC only, 10:1, 5:1, 1:1, 1:5, 1:10, and OB only were seeded in each well at a concentration of 7500 cells/150 μL. Media changes were performed daily with 150 μL. Cells were stained with calcein AM before imaging (n 3 4 samples).

Fluorescence microscopy images were obtained at days 1, 3, and 7 at 10× using an inverted microscope (Nikon). Five to 10 ran dom images were taken of each sample. Image analysis was performed by pattern recognition as described by Luesch et al.[18] In this method, a number was assigned to the angiogenesis pattern present in each image. Individual cells that were well separated were assigned 0 point. Migrating cells that were aligning were assigned 1 point, capillary tubes without sprouting were assigned 2 points, sprouting of new capillary tubes were assigned 3 points, the forma tion of closed polygons were assigned 4 points, and if complex mesh like structures were present, 5 points were assigned.[18]

Matrigel Angiogenesis Assay

The ability of the different coculture ratios to form vasculogenic networks was also evaluated using a Matrigel, a provasculogenic substrate (Millipore, Billerica, MA). Two sets of experiments were done in Matrigel. The first experiment had the same total cell number in each well for each group so the seeding density was the same. A 150 μL of the cell solution with a total cell number of 7500 cells per well were added to each well. The second experiment had 7500 ECs per well and OBs were added to achieve the desired coculture ratio. To perform the assay, 50 μL of EC matrix solution was added to pre cooled 96 well tissue culture plates and incubated at 37°C for 1 hour. human umbilical vein endothelial cells and OBs were trypsinized and resuspended in coculture media in the following coculture ratios (EC/OB): EC only, 5:1, 1:1, 1:5, and OB only. The 1:10 and 10:1 ratios were not done because based on preliminary experiments, the number of cells required to achieve these ratios was not feasible under the re straints of the assay and well sizes. For both experiments, the cells were incubated for 6 hours at 37°C before imaging. Imaging was performed using phase contrast microscopy (Nikon) at 10×. Five random images were taken of each sample. Image analysis was performed by pattern recognition counts as was done with the collagen assay (n 3 samples).

Statistics

Statistics were performed using 1 way analysis of variance with significance determined at $P < 0.05$. Results are reported as mean (SD).

RESULTS

Cocultures on Polystyrene

The direct coculture 1:5 and 1:10 ratios on polystyrene were the only groups to show evidence of cell organization and minerali zation. Endothelial cells clustered together and a separation between the EC and OB could be seen starting at day 3 in these 2 groups (Fig. 1A). This organization of cells was also seen in the 1:5 and 1:10 groups on the collagen assay starting at day 7 (Fig. 2). In the 1:5 and 1:10 polystyrene direct coculture groups at day 10, there was evidence of nodule like formations with OBs situated radially around the circular formation (Fig. 1B) and in subsequent days there was continued nodule formation and mineralization patches present with no evidence of cell death (Fig. 1C). Osteoblast monocultures did not form nodule like formations or patches and the cells remained elongated and parallel to each other through day 21 (Fig. 1D).

Endothelial cell monocultures reached confluence and the cells started to become apoptotic at day 14 and did not survive to day 21 (Fig. 1E). The 10:1 and 5:1 direct coculture groups showed pro liferation of both cell types throughout the 21 days but the ECs and OBs grew on top of each other, forming a mesh like network starting at day 14 with no nodule formation (Fig. 1F). The 1:1 group also had ECs and OBs growing on top of each other, but the cell layer partially

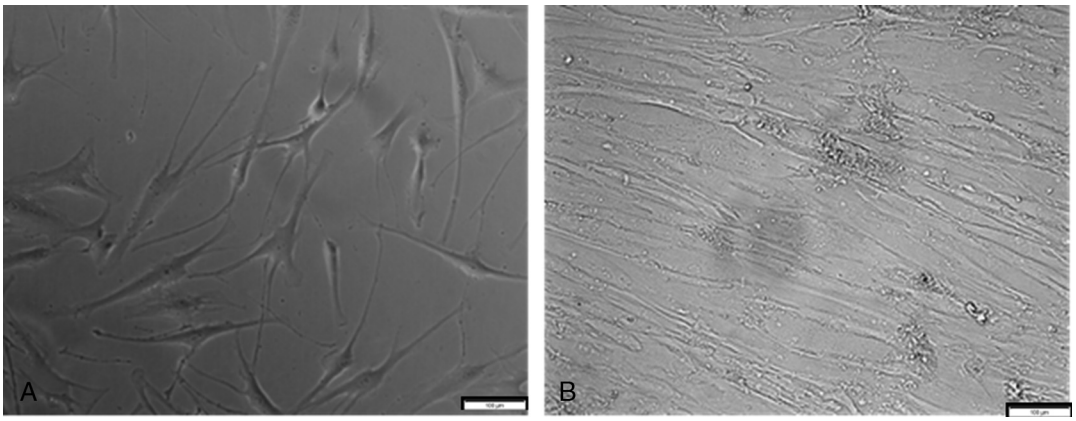

FIGURE 3. Indirect polystyrene cocultures: (A) 5:1 indirect coculture with flattened OBs with multiple filopodia on day 3 and (B) 5:1 indirect coculture on day 21.

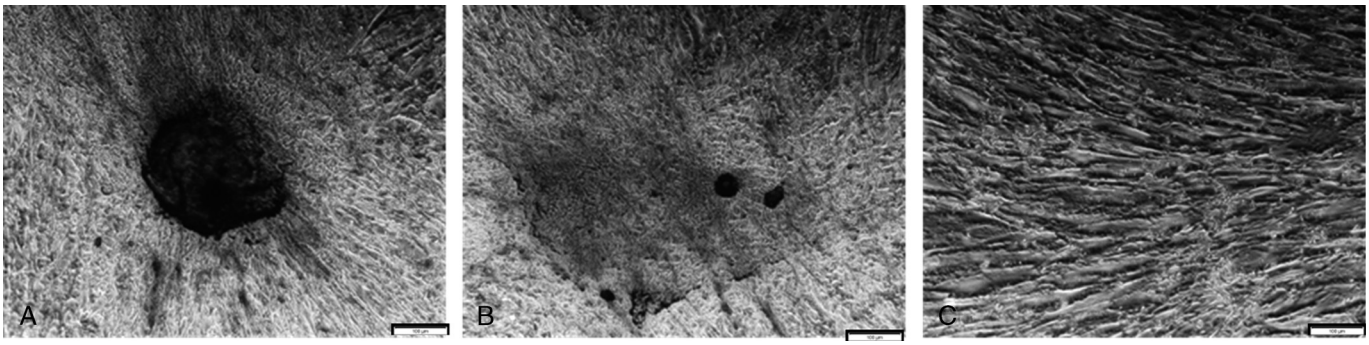

FIGURE 4. Alizarin Red S staining on day 21: (A) mineralization nodule in 1:5 direct coculture, (B) mineralization patch in 1:5 direct coculture, and (C) no mineralization in OB monoculture.

lifted off the substrate on day 10. From days 10 to 21, mostly OBs remained in the 1:1 culture with a few EC clusters present.

In the indirect coculture group, the OBs were spaced far apart in groups 10:1, 5:1, and 1:1 and the cells took on a flattened ap pearance with multiple extended filopodia on days 1, 3, and 7 (Fig. 3A). There were no noticeable differences in OB morphology between groups 10:1, 5:1, 1:1, 1:5, 1:10, and OB on and after day 10. From days 10 to 21, the OBs in all groups remained elongated and parallel to each other (Fig. 3B).

Alizarin Red S Staining

Alizarin Red staining was performed at day 21 for the poly styrene direct and indirect cocultures. The staining showed mineral ization nodules and patches on the 1:5 and 1:10 direct coculture ratios (Fig. 4A C). No mineralization was seen on the other direct coculture ratios or on any of the indirect coculture ratios.

Alkaline Phosphatase Assay

Although at day 1, there was no difference in alkaline phos phatase secretion between any of the direct coculture groups, by day 7, the 1:5 direct coculture group secreted significantly more alkaline phosphatase than the other groups and remained the highest through day 18. At day 12, 1:10 began to increase its secretion of alkaline phosphatase and remained at levels close to 1:5 for the rest of the culture period. On day 21, the amount of alkaline phosphate dropped back to or below day 1 levels in all groups (Fig. 5A).

In the indirect coculture groups, there were no significant differences in alkaline phosphatase secretion between the groups af ter day 1. On day 1, there was very little alkaline phosphatase se creted in the 1:5, 1:10, and OB group which does not correlate with the direct culture values and could be due to error; but on day 3, these groups had a levels of alkaline phosphatase to that secreted by the other groups (Fig. 5B).

VEGF Enzyme-Linked Immunosorbent Analysis

Osteoblast monocultures secreted VEGF over 21 days and there was no significant difference between the indirect and direct groups, showing that the presence of the transwell membrane in the indirect group did not alter OB VEGF secretion. The EC monocultures did not secrete any detectable levels of VEGF in direct or indirect cultures. In the direct contact cocultures, VEGF was detected only in the OB, 1:10, and 1:15 groups. After day 1, the amount of VEGF secreted decreased in the 1:5 and 1:10 direct coculture groups, but started increasing at day 7. By day 21, the 1:5 direct coculture ratio VEGF secretion exceeded that of the OB group. Vascular endothelial growth factor was also present in the 1:5 and 1:10 indirect coculture ratios on day 1 but was inhibited afterward and did not reappear in detectable levels until day 18. For all coculture groups, there is significantly more VEGF secreted than in the indirect groups (Fig. 6).

Collagen Assay

The pattern recognition count defines the visual vasculogenic networks seen in each image of the well. The higher the number, the more vasculogenic progression that was present. At day 1, the vasculogenic response was dependent on EC density. The groups

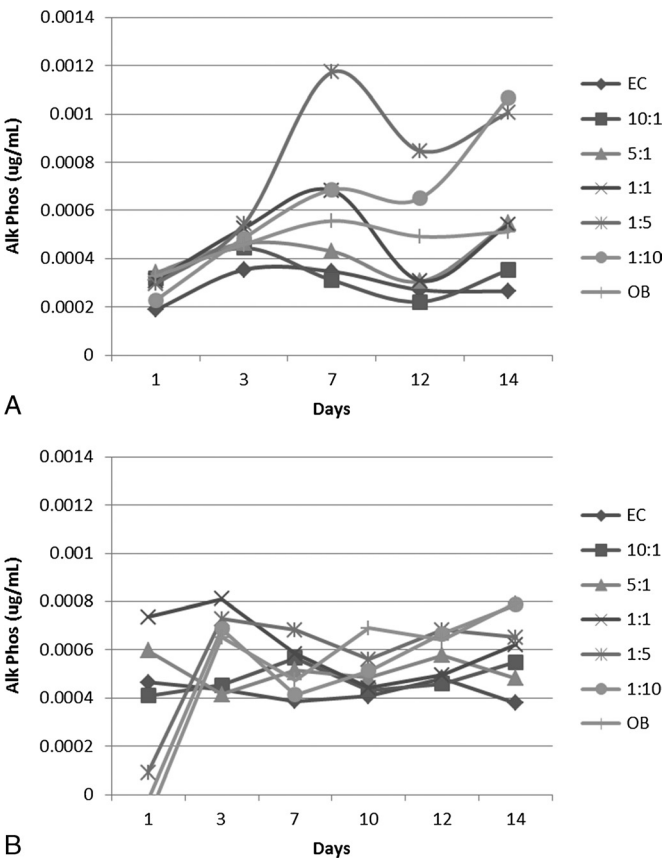

FIGURE 5. Alkaline phosphatase assay. A, Direct coculture. Increase in secretion for 1:5 at day 7 and at day 12 for 1:10, correlating with formation of mineralization nodules. B, Indirect coculture showed constant secretion of alkaline phosphatase throughout the culture period for all groups (n = 3, $P < 0.05$).

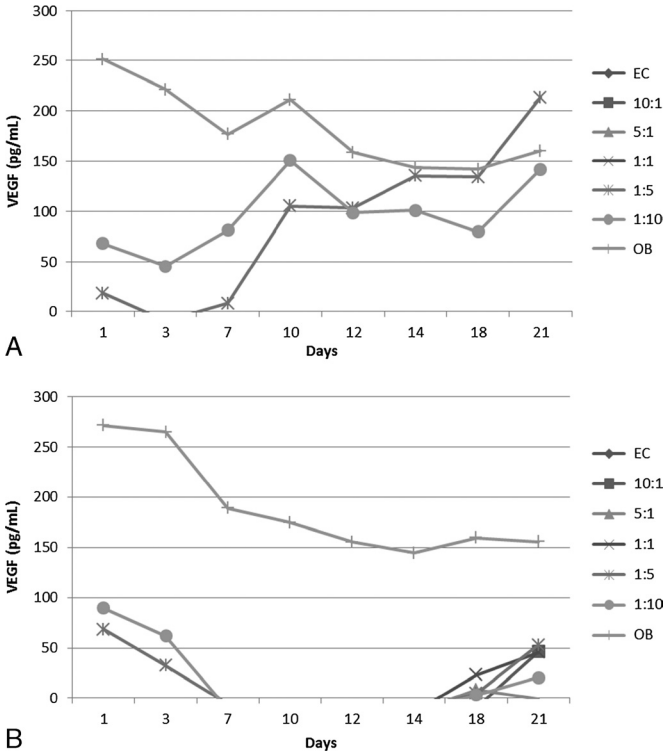

FIGURE 6. VEGF assay. A, Direct coculture. No VEGF is secreted except in groups OB, 1:5, and 1:10. At day 21, 1:5 had significantly more VEGF than all groups except 1:10 and OB. B, Indirect coculture. OB VEGF secretion is significantly higher than all the coculture and EC groups (n = 3, $P < 0.05$).

with more ECs had more cells lining up and forming capillary like formations. At day 3, EC, 10:1, and 5:1 had significantly more vasculogenic activity than the rest of the groups. At day 7, 5:1 had significantly more vasculogenic formations than 1:10, 1:5, and EC. Endothelial cell and 10:1 groups peaked at day 3 and decreased on days 7 and 10 after they were confluent in the culture showing evi dence of contact inhibition. The 1:1, 1:5, and 1:10 continue to show increased vasculogenic network formation up to day 7. Throughout the 7 days, the OB group did not have any vasculogenic formations and it, along with 1:10 and 1:5, had the lowest values (Fig. 7). On day 7, the cells in the 1:5 and 1:10 groups clustered together into circular formations with a lining of ECs (Fig. 2).

Matrigel Angiogenesis Assay

The Matrigel angiogenesis assay was performed to determine if the presence of OBs was inhibitory to vasculogenic performance because ECs are able to form capillary networks in Matrigel without any additional vasculogenic factors. When the wells were seeded so that each well had the same number of ECs (Equal EC group), there were no significant differences between the groups (Fig. 8). When the wells were seeded so that there was an equal number of total cells seeded (Seeding density group), resulting in a different number of EC seeded in each well, the EC group had significantly more vas culogenic network formation than the 1:1 and 1:5 groups. The EC group also had more network formation than the 5:1 group, but it did not result in a statistically significant difference. There was also a significant difference between the 5:1 and 1:1 groups (Fig. 9). The results show that in Matrigel, the vasculogenic response is dependent on the number of ECs present in the culture and that OBs do not have an inhibitory effect on angiogenesis when in a provasculogenic environment.

DISCUSSION

Successful bone regeneration consists of both bone formation and vascularization, and cocultures of cells have been increasingly explored to achieve this.[19] Various types of cells have been cocultured with ECs, such as mesenchymal stem cells,[20] stromal cells,[17] fibro blasts,[21,22] and human primary cells.[8,12,13] An advantage of using human primary cells rather than stem cells is that differentiation of the cells through the use of specialized media with osteogenic factors is not required to achieve mineralization, which could translate into eliminating the need for exogenous growth factors such as bone mor phogenetic protein 2. Many studies focus on the behavior of the cells in coculture that have an equal amount of ECs and OBs,[11,16] but it is apparent that the ratio of the cocultured cells is an important factor that should be considered when designing implants.

Vasculogenesis

The vasculogenic response in collagen seems to be dependent on having enough ECs to form the networks, but not too many that the field becomes confluent and contact inhibition occurs, which is what is seen in group 10:1 and EC at days 7 and 10. The 5:1 coculture ratio, followed by 1:1, have a ratio of ECs to OBs which fosters ECs lining up and forming capillary like formation without

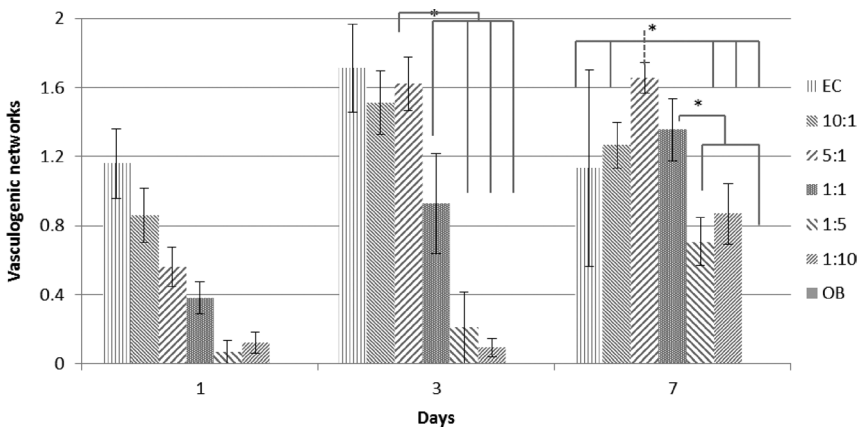

FIGURE 7. Collagen assay vasculogenic networks: 5:1 and 1:1 showed the highest vasculogenic networks for day 7; 5:1 had significantly more vasculogenesis than all groups except 1:1 [mean (SE), n = 3-4, $P < 0.05$].

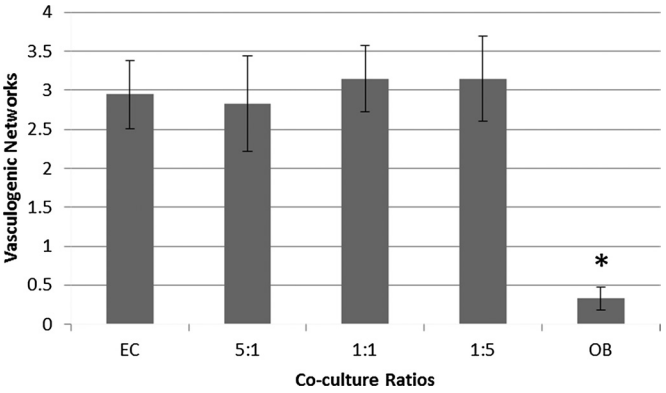

FIGURE 8. Matrigel angiogenesis assay of the Equal EC group. The number of vasculogenic networks was significantly lower for the OB group. There were no differences between the other groups (n = 3, $P < 0.05$).

the culture overwhelming the OBs and becoming confluent and losing organization. The 1:5 and 1:10 had more vasculogenic formations in collagen than expected at days 7 and 10 because they had the fewest number of ECs in culture. Although the values were not as high as those for EC, 10:1, and 5:1, these cultures demonstrated EC organization which may have become more apparent and significant if the culture period was extended further. Unfortunately, the culture could not be extended past 10 days because the collagen delaminated off of the wells at day 12. The response in these culture groups may be a result of increased VEGF secretion from the OBs which was seen in the polystyrene experiments.

Endothelial cells do not secrete VEGF in nonhypoxic conditions so the VEGF response seen in this set of experiments is only from the OBs.[23] At day 1, measurable amounts of VEGF in both the indirect and direct culture groups were seen only in the OB monoculture and the 1:5 and 1:10 cocultures. The increase in VEGF in the direct cocultures seen at day 7 for 1:5 and 1:10, which coincides with the formation of nodules, can be explained by the EC and OB crosstalk that has been discussed in other studies which occurs with

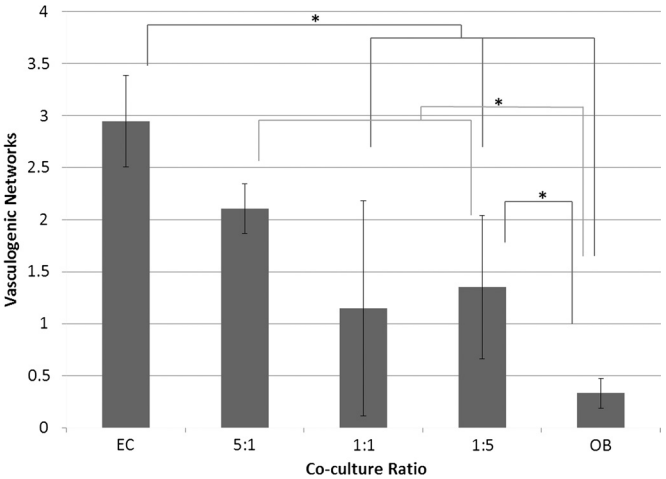

FIGURE 9. Matrigel angiogenesis assay of the Equal Seeding Density group. EC had the highest vasculogenic pattern counts and the number of ECs present is proportional to the pattern counts seen for the different groups with the exception of 1:5 (n = 3, $P < 0.05$).

direct contact between the cells.[10,15] As the ECs interact with the OBs, a release of paracrine factors occurs which has been shown to increase mineralization and, in turn, increase OB secretion and expression of angiogenic factors.

The Matrigel assay provided another way to look at angiogenesis in a gel like matrix. Endothelial cell monocultures are able to form vasculogenic networks due to the angiogenic factors in the Matrigel. The results show that the amount of capillary formation in Matrigel is roughly proportional to the number of ECs seeded. However, the 1:5 group had similar numbers of vasculogenic network formations as the other coculture groups with more ECs, which was not expected. Because the components in the Matrigel speed up the vasculogenesis process, they could also be increasing the effect of the OBs on the ECs in that ratio. In the collagen and polystyrene cultures, the ECs in the 1:5 ratio aggregate which also occurs in the Matrigel. The images of the 1:5 ratio show that there is aggregation of the cells at the junction points of the networks which may be serving as a nidus for the capillary tubules. Additionally, the secretion of VEGF was higher in the 1:5 coculture than in the other coculture ratios which may aid in an increased amount of vasculogenesis. When the OBs were cultured in the Matrigel by themselves, the cells mostly stayed separated with only a few cells migrating together. No filopodia were seen in these cultures so the extended capillary tubules seen in 1:5 were from the ECs and not OB filopodia.

Mineralization

Vascularization is an important part of bone regeneration and healing, not only because it provides a conduit for oxygen and nutrients but also because it helps activate bone morphogenic protein mediated healing processes. In studies in which angiogenesis was inhibited, there was significantly reduced bone healing in vivo.[24] Vascular endothelial growth factor is secreted in a differentiation dependent manner during osteogenesis and is an essential coordinator in endochondral bone formation. It also has a role in OB differentiation and mineralization.[25–27] These factors may also have a part in the increased mineralization present in the 1:5 coculture, but in vivo studies need to be performed to fully explore this phenomenon.

It was hypothesized that a coculture of ECs and OBs where more OBs than ECs are present would result in increased mineralization compared to cultures with an equal amount or more ECs than OBs. In this study, mineralization was seen only in the 1:5 and 1:10 direct cocultures with 1:5 having more mineralization nodules and patches than the 1:10 culture. This is also reflected in the alkaline phosphatase assay where the highest amount of alkaline phosphatase was produced by the 1:5 direct ratio group followed by 1:10 direct ratio group. Because mineralization occurs only in the direct coculture and not in the indirect coculture with the same cell ratios, it suggests that there are more than paracrine effects at work. Multiple groups have demonstrated that in cocultures, the contact between the cells is an important part of the cellular response.[6,7,28] When the cells do not have the opportunity to interact with each other through physical contact, mineralization is not promoted. When cells are exposed to only cultured media and are not able to form cell to cell contacts, an increase in expression of alkaline phosphatase is also not seen.[6,7,28] Alkaline phosphatase secretion is an indicator of active calcium deposition and is a byproduct of OB activity. Its secretion has been shown to be dependent on endothelial contact with OBs with Connexin43 as the gap junction involved in the increase in alkaline phosphatase expression in the cocultures. This secretion is not only dependent on the presence of contact but also on how much contact and how long the different cell types have with each other.[29–31] In the groups with more OBs than ECs, the most alkaline phosphatase secretion was seen from days 7 to 14, after which there was a decrease in secretion. The EC monoculture group did not have a significant rise and fall of the alkaline phosphatase levels throughout the culture period for both the indirect and direct

cultures. This reflects a constant secretion of alkaline phosphatase from ECs, whereas the OB secretion of alkaline phosphatase is related to the mineralization activity and OB differentiation.

The choice of culture medium is important because minera lization occurs in OB monocultures in osteogenic media containing β glycerophosphate and dexamethasone. In this study, the media used do not contain dexamethasone or β glycerophosphate and, therefore, did not promote mineralization in OB monocultures after 21 days of culture. Because of this, mineralization seen in the cul tures can be attributed to the effects of the coculture and not the culture media. The use of primary differentiated cells in this study is also important. Ma et al[17] have also investigated the effect of coculture ratios on osteogenic and vasculogenic outcomes with human marrow stromal cells; however, osteogenic media were required to differenti ate the cells and thereby induce mineralization. In culture medium similar to what was used in this study, no mineralization was seen.

CONCLUSIONS

To improve vasculogenesis in human primary EC and bone derived OB cocultures, ratios of 5:1 or 1:1 should be used; but to increase mineralization, the 1:5 direct coculture ratio, followed by the 1:10 direct coculture ratio, should be used. This information can be used to develop future tissue engineering scaffold systems which use preseeded primary cells or even composite scaffolds for bone seg mental defects which have an environment which would support native OB migration into the scaffold. Additionally, this information can be used in systems to augment and expand the use of bone grafts.

For complex tissues, such as bone, a vascular network needs to be established to support cell infiltration and growth into central areas of the scaffold. On the basis of this study, areas needing more vascularization could be implanted with a 5:1 coculture and the areas where bone formation is desired can be implanted with a 1:5 coculture. In these cultures, there is enough of 1 cell type to have enough of an effect on the other cell type, but not sufficient to overwhelm and overtake the culture. The study confirms that contact between the ECs and OBs is important and that the simple addition of growth factors into the milieu may not be enough to induce vas culogenesis and mineralization.

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