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The stimulation of angiogenesis and collagen deposition by copper

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ABSTRACT

Copper is known to trigger endothelial cells towards angiogenesis. Different approaches have been investigated to develop vascularisation in biomaterials. The angiogenic and healing potential of copper ions in combination with two major angiogenic factors was examined. A 3D culture system in which, under stimulation by FGF-2 and to a lesser degree with VEGF, endothelial cells assembled into structures resembling to an angiogenic process was used. The combination of $CuSO_4$ with increasing doses of VEGF or FGF-2 enhanced the complexity of angiogenic networks in a significant manner. *In vivo* studies were also conducted by incorporating FGF-2 with $CuSO_4$ in a cylindrical collagen-based scaffold. $CuSO_4$ enhanced significantly the invasion of microvessel compared to control implants and to 20 ng FGF-2 ± $CuSO_4$. Vascular infiltration was also significantly improved by combination of $CuSO_4$ with FGF-2, compared to FGF-2 alone (0.2 and 1 μg). Nevertheless, in comparison with $CuSO_4$ alone, there was a significant increase only with 1 μg of FGF-2 combined with $CuSO_4$. Significantly, collagen fiber deposition was enhanced following the combinatory loading in comparison to that with FGF-2 alone but not with $CuSO_4$ only. Thus, copper associated with growth factors may have synergistic effects which are highly attractive in the fields of tissue engineering (e.g., bone) and biomaterials.

1. Introduction

Formation of a blood supply represents an important phase of wound healing as well as in tissue ingrowth that occurs in porous implants. It is also important to enhance cell survival in tissue engineered devices, and vascularisation in ischemic tissue such as myocardium as therapeutic angiogenesis [1]. Different approaches have been proposed to stimulate or facilitate angiogenesis/vasculogenesis; amongst them, the delivery of vascular growth factors (GFs) such as VEGF often released from a polymer [2,3]. Although the availability and efficacy of human recombinant GFs is now well established, their clinical applications may be limited by the large amount needed associated with long term medical costs, and their potentials to raise some undesirable effects at repeated doses.

In the body, trace elements of stable inorganic ions, especially metal ions, act as enzyme co-factors (e.g., metalloproteinases), and stimulate cell signaling pathways towards tissue equilibrium. Copper ions are particularly involved in the activity of several transcription factors (via HIF-1 and proline hydroxylase) and bind to cell membrane releasing complex [4–6], facilitating release of GFs and cytokines from producing cells [7]. Moreover, copper has been shown to stimulate endothelial cell proliferation and enhance angiogenesis *in vitro* [8,9]. *In vivo* copper decreased the risk of ischemia in skin flaps and induced a vascularised capsule around a crosslinked hyaluronic acid-composed hydrogel [10,11]. Recently, we reported that low doses of copper sulphate ($CuSO_4$) loaded in a macropore of a 3D printed bioceramic scaffold facilitated implant vascularisation and wound tissue ingrowth which was further enhanced by combination with a low dose of VEGF [12,13].

The aim of the present study was to investigate the *in vitro* and *in vivo* effects of combining $CuSO_4$ with angiogenic GFs. A fibrin gel was used to investigate the behaviour of endothelial cells in a 3D angiogenesis culture system in the presence of different doses of $CuSO_4$ with or without FGF-2 or VEGF. *In vivo* studies were conducted using a tubular collagen-based scaffold in which an optimal dose of $CuSO_4$ was only loaded or in combination with increasing concentrations of FGF-2.

2. Materials and methods

2.1. In vitro study

2.1.1. Endothelial cells

Human endothelial cells derived from umbilical cord vein (HUVECs) between passages 3 and 6 were propagated in Medium 199 (M199, Sigma–Aldrich, Inc., St.

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Louis, MO) supplemented with 10% foetal bovine serum (FBS, Invitrogen Co., Burlington, Ontario), in the presence of endothelial cell growth supplement (ECGS, 20 μg/ml, BD Biosciences, Bedford, MA), heparin (90 μg/ml, Sigma–Aldrich), L-glutamine (2 mM, Sigma–Aldrich), and antibiotics/antimycotic (Penicillin/Streptomycin and Amphotericin B solution, Invitrogen Co.).

2.1.2. 3D angiogenesis system

Endothelial cells were embedded in a fibrin gel according to a method previously described [14]. Briefly, a fibrinogen solution (3 mg/ml in Hank's Balanced Salt Solution) was mixed with 2×10^5 endothelial cells per ml. Each well of a 24-wells plate was filled with 500 μl of the cell suspension; and 15 μl of thrombin (50 U/ml; Warner-Lambert Canada Inc., Scaborough, Ontario) solution was promptly added to each well to polymerize fibrinogen into fibrin gel (10 mn at 37 °C). The fibrin matrices were then covered with 1 ml serum-supplemented medium (i.e., M199 with 10% FBS, 2 mM L-glutamine, 90 μg/ml heparin, without any growth supplement). The effects of increasing amount of $CuSO_4$ (10 ng–50 μg) was investigated as well as that of rhFGF-2 (1–50 ng/ml, BD Biosciences) or $rhVEGF_{165}$ (0.1–2 ng/ml; R&D Systems, Minneapolis, Minnesota). Cell behaviour was periodically observed by phase contrast for 12 day culture period.

2.1.3. Quantification of structures formed by endothelial cells

After 12 days of culture, gels were fixed in 3.7% formaldehyde, then rinsed in phosphate buffered saline solution (PBS), and stained with Toluidine Blue (1% in water) for 2 mn and then extensively rinsed in water. Blue-stained gels were directly observed without phase contrast (*see* Supplementary figure I). Micrographs were taken at low magnification (×4 objective) through a video camera (Q-Imaging, Burnaby, British Columbia) connected to an inverted microscope with a long working distance condenser (Diaphot, Nikon Canada). Five microscopic fields per well (one at the center and four at opposite corners) were recorded. Using Image J software, (Scion Corporation, Frederick, Maryland), blue-stained endothelial cells and structures resembling to angiogenic processes were threshold on each image of microscopic field. An area was traced to eliminate any artefact of threshold. Using the measurement tool, threshold regions of interest (i.e., bluish structures) were measured and reported to the selected surface area. Values were expressed in percentages of this area. Three specimens for each condition were quantified on five microscopic fields per specimen and the experiments were repeated at least twice ($n \geq 30$).

2.1.4. Statistical analyses

Statistic analyses of cell culture assessments were performed using the one-way analysis of variance (SigmaStat®, Systat Software, Inc., Point Richmond, CA) and the Student–Newman–Keuls Method as all pairwise multiple comparison procedures with a p value of 0.05.

2.2. In vivo study

2.2.1. Tubular collagen-based scaffolds

Scaffolds with a cylindrical shape and a central cavity closed at one end was made from insoluble type I collagen by freeze-drying a 1% collagen dispersion in a mold as previously reported [15]. Freeze-dried scaffolds were then immersed in trifluoroacetic acid (TFA; Sigma Chemical Co.) for 1 h at room temperature followed by a vacuum evaporation in the presence of KOH, as previously described [16]. After several washes in ddH_2O, scaffolds were then freeze-dried and treated by a severe dehydration (for 2 days at 110 °C under a 100 mTorrs vacuum).

Prior to implantation, each scaffold was loaded with 560 ng CuSO4 combined or not with 20, 200 and 1000 ng FGF-2 diluted in PBS. In sterile conditions, a 20 μl mixed solution was introduced with a thin tip into the central cavity of pre-wetted scaffold. This allows concentrating the compound(s) to be tested in the middle of the implant. Control implants were loaded with 20 μl of vehicle solution.

2.2.2. Implantation

The implantations were performed in CD1 mice after approval by our local Institutional Animal Care Committee and in agreement with the guidelines of the Canadian Council for Animal Care. Scaffolds were implanted subcutaneously in the thigh close to the inguinal fat pad. The skin incision was performed on the lateral side of the limb to avoid any interference with the healing process within the scaffold. The incision was then sutured with non-resorbable polypropylene monofilament. For each condition, at least three implants were investigated. After 30 days, implants were retrieved with their nearby adherent tissues and fixed in zinc fixative for regular staining and immunohistochemistry.

2.2.3. Observation of the cell/tissue infiltration

Specimens were cut longitudinally along the central cavity before being processed for histological sections in paraffin. Tissue sections were stained with Hematoxylin, Eosin, and Safran (HES) for regular observation of tissue infiltration.

2.2.4. Microvessel formation

Immunohistochemistry was processed on unstained zinc-fixed tissue sections. Sections were deparaffinised and rehydrated. Tissues were treated with 3% hydrogen peroxide, and then blocked with a blocking reagent containing rat serum in PBS (Chemicon International, Cedarlane, Ontario). After a brief washing, primary antibody towards murine CD31 (rat anti-mouse CD31 monoclonal antibody, BD Pharmingen, Invitrogen, Ontario) was applied at 1/50 dilution. After 2 h of incubation at room temperature, tissues were rinsed (3x in TRIS-buffered saline (TBS) solution containing 0.1% Tween20), and a secondary antibody (Biotin-conjugated goat anti-rat Ig polyclonal antibody, BD Pharmingen, Invitrogen, Ontario) was incubated at a 1/50 dilution. After incubation, tissues were rinsed (3x TBS), and a fresh solution of Streptavidin-horseradish peroxidase 3,3′diaminobenzidine was applied to reveal the antibody reactivity, followed by several rinses. Slides were washed in water, mounted in a TRIS glycerol mounting fluid, and observed under microscopy without counterstaining. CD31+ microvessels was quantified in the scaffold porous structure by measuring the area occupied by the capillaries relative to the total surface area occupied by the scaffold in each microscopic field (10X objective) and expressed in area fraction. Four microscopic fields were analyzed for each implant with three implants per condition, excluding the area occupied by the scaffold central cavity. Image J software (Scion Co., Frederick, Maryland) was used for measurements.

2.2.5. Connective tissue formation

Tissue sections were stained with picro-sirius in order to observe the newly deposited and organized collagen fibers under polarized light which appeared birefringent in contrast with the implanted (exogenous) collagen (*see* Supplementary figure II). Recorded images (10x objective) were analyzed with the Image J analysis system. Quantification was performed by reporting the surface area occupied by the birefringent collagen fibers over the total surface of the void spaces within the scaffold and expressed as percentage. Five microscopic fields were analyzed for each implant, excluding the area occupied by the scaffold central cavity.

2.2.6. Statistical analyses

Statistic analyses of *in vivo* assessments were performed using the one-way ANOVA test and the Fisher LSD method as multiple comparison procedure with a p value of 0.05.

3. Results

3.1. In vitro studies

In control cultures, most endothelial cells remained isolated and rather round with very few initial structures (Fig. 1 and Supplementary figure 1). Similar observations were found in the presence of low doses of $CuSO_4$ (100–1000 ng/ml). In the presence of increasing amount of $CuSO_4$ alone, the extension of endothelial cells into tiny cord- and tube-like structures behaved in bell-shape curve with a peak at 50 μg/ml which was significantly higher than the values for 25 ($p \leq 0.01$) and 100 ($p < 0.013$) μg/ml $CuSO_4$ and for the control cultures ($p < 0.011$) (Fig. 1A). Lower doses (10–1000 ng/ml) were also tested for which the values were close to those in control cultures (data not shown). As these structures were most visible at 50 μg/ml $CuSO_4$ concentration, subsequent cell culture experiments were performed with this optimal dose.

The addition of increasing amount of VEGF did not significantly enhance angiogenesis compared to control cultures, and there was no significant difference between the different doses of VEGF alone (Fig. 1B). These values were significantly lower than that of $CuSO_4$ alone (2 ng/ml VEGF $p = 0.002$; 10 ng/ml VEGF $p < 0.009$; and 25 ng/ml VEGF $p < 0.003$). Conversely, the presence of increasing concentration of VEGF with 50 μg/ml $CuSO_4$ significantly induced the formation of tubular structures resembling to an angiogenic process (2 ng/ml VEGF $p = 0.008$; 10 ng/ml VEGF $p < 0.001$; and 25 ng/ml VEGF $p < 0.001$ respectively compared to $CuSO_4$ alone) (Fig. 1B & D), Similar difference was also found by comparison with increasing doses of VEGF alone ($p < 0.001$ at any doses) and with control medium ($p = 0.002$).

In the presence of FGF-2 alone the extension of tube-like structures with lumens lined by endothelial cells increased significantly as a function of increasing doses of FGF-2 compared to control cultures (10 ng/ml FGF-2 $p = 0.016$; 25 ng/ml FGF-2 $p < 0.001$; 50 ng/ml FGF-2 $p < 0.001$) and to $CuSO_4$ alone ($p = 0.011$) (Fig. 1C & D). The combination of $CuSO_4$ with increasing doses of FGF-2 resulted into a complex network with lumen and intercommunications. In those conditions, higher and significant values were