Inhibition of The Fracture Healing Process in Smokers: Deregulation of Cellular and Molecular Milieu in Tibial Fracture Micro-Environment

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ABSTRACT

Introduction: Tobacco smoking has been shown to have a detrimental impact on fracture healing and is often implicated in non- and delayed-union of bone. Whilst numerous studies have concentrated on the demographic and clinical manifestations of fracture healing and smoking, very little analyses have been undertaken at the biochemical level. **Aims:** This research project will assess the impact of smoking on the cellular and molecular mechanisms of bone healing by analysing human tibial fracture haematomas using a variety of laboratory techniques. **Materials and methods:** *Cell culture:* Fracture haematomas (~2.0 mL) were collected from anaesthetised patients (*n*=48), who had sustained a fracture of the tibia. The semisolid material was explanted into 25 cm^2 non-coated tissue culture flasks and allowed to clot. Complete culture media was prepared (Iscove's Modified Dulbecco's Media (IMDM) containing 10% Fetal Calf Serum (FCS), 100 g mL^{-1} Streptomycin, 100 U mL^{-1} Penicillin antibiotic, 0.25 g mL^{-1} amphotericin B and 2.0 mM L-glutamine (all Sigma-Aldrich, Gillingham)) and pipetted into the vessels, which were then placed in an incubator (37˚C; humidified 5% CO_2). After characterisation via immunocytochemistry (using antigen-specific markers to CD29, CD44, CD73, CD105, CD166 and CD34), isolated cells were counted using flow cytometry and proliferation rates were compared between non-smokers and smokers. Cigarette smoke extract (CSE) was manufactured based on the methods of Bernhard *et al.* (2004), in order to synthesise an *in vitro* smoking environment. Cells harvested from non-smokers (*n*=10) were cultured and infused with the CSE (2.5%) and proliferation and cell recovery rates were compared between the treated and untreated groups. Cellular nitric oxide levels and necrosis rates (using propidium iodide for the latter) were also compared in a similar way. An MTT assay was conducted to assess response to various concentrations of BMP-2 (10, 100 and 500 ng mL^{-1}) infusion in cell cultures. *Molecular assays:* Serum was extracted from the haematomas and resolved on 16% acrylamide gels before staining with either Coomassie blue or silver nitrate. The resolved gels were photographed using a Kodak gel imaging system and unknown bands were identified using MALDI-TOF. Intracellular TGF- and IL-6 were probed using antibodies, which were then analysed via flow cytometry in order to confirm the presence of these acute-phase proteins. In additional experiments, the ELISA was undertaken to quantify and compare the amounts of VEGF and IL-6 in haematoma serum and lysed fracture cells respectively. Biochemical testing on serum for b-ALP, albumin and total protein amounts in fracture serum was carried out, comparing smoker (*n*=10) and non-smoker (*n*=10) levels. **Results:** *Cell*

culture: Spindle-shaped, fibroblast-like cells were visible in the primary culture, which were expanded through at least 10 further passages. The immunostaining of cells suggested a mesenchymal stem cell (MSC) lineage (CD29+, CD44+, CD73+, CD105+, CD166+, CD34-). Absolute cell counting using flow cytometry revealed marked proliferation of cells after 3 passages of culture. There was a reduction in the rate of proliferation of MSCs in smokers over 2 passages compared with non-smokers (-20%). A 5-day proliferation study in the CSE-treated vs. untreated cells showed a reduction in the rate of doubling in the treated group (-40% $p<0.05$). Cells showed a recovery response after CSE was withdrawn from culture, but was not significant. Cell necrosis analysis of CSE-treated vs. untreated cells showed that the CSE-treated cultures had a higher rate of necrosis than the untreated cells (CSE-treated 14.37% vs. untreated 7.98% $p<0.05$). Nitric oxide levels were lower in the CSE-treated cells (CSE-treated 3.0 M vs. untreated 3.6 M; $p<0.05$). MTT assay; BMP-2 infusions all improved cell viability compared to the non-infused cells, with a BMP-2 concentration of 10 ng mL^{-1} increasing cell viability the most, though not significantly (+23%; $p>0.05$). *Molecular assay:* After staining, SDS-PAGE gels showed numerous bands of serum proteins and the haematoma serum lanes displayed unknown proteins which were later found to be haptoglobin (~20 kDa) and haemoglobin (~14 kDa) chains via mass spectrometry (MALDI-TOF). Cells were strongly positive (>96%) for the intracellular protein markers TGF- and IL-6. The VEGF-A (serum) and IL-6 (cells) preliminary data from the ELISA revealed a reduction of these acute-phase proteins in patients who were smokers (VEGF-A -10%; IL-6 -15%). In the biochemical assays, albumin was reduced in smokers' serum (-13%, $p<0.05$), whereas b-ALP was raised, albeit not significantly (+20%; $p>0.05$) and total protein lowered, which was significant (-12%; $p<0.01$). **Conclusions:** The haematoma cultures produced colonies of adherent fibroblast-like cells that were of MSC lineage. With the exception of a residual haemolysis-derived haptoglobin band, SDS-PAGE did not appear to show an over-expression of acute phase proteins, although it was useful for the characterisation of the fracture milieu, in that it was suggestive of a consistently haemolysed haematoma. The effect of smoking on bone fracture healing, therefore, appears to contribute to the inhibition of MSC proliferation, angiogenesis and the acute phase stress response. Cigarette smoke was also shown to cause excessive necrosis and reduce the amount of NO in those MSC cultures treated with CSE, which may indicate reduced vasodilatation to the fracture site. Bone alkaline phosphatase (b-ALP) was raised in the fracture serum of smokers, suggesting abnormally-high bone turnover, whilst the reduction of total protein and serum albumin likely indicates a lowered capacity for acute-phase protein synthesis and Ca^{2+} transport in these patients. Exogenous BMP may be indicated for improving malunion in smokers.