Role of Ozone/Oxygen in Fibroblast Growth Factor Activation. Discovering the Facts

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SUMMARY - *Basic fibroblast growth factor (bFGF) is a pleiotropic mitogen which plays an important role in cell growth, differentiation, migration and survival in different cells and organ systems. Application of bFGF has been shown to promote cellular proliferation and collagen synthesis in vivo. FGF is markedly up-regulated following bone or tendon injury and active at multiple stages of the healing stimulation process, local blood circulation, lipolysis and smooth muscle. Looking at the physical and chemical properties of the ozone molecule, the present work deals with its possible therapeutic action as an FGF activator. Incubation (2 h) of platelet-rich plasma with 80 µg/mL O_2/O_3 increases the basal concentration of FGF by approximately 600%. This fact in combination with previous demonstration of the stimulating action of O_3, releasing other platelet factors may potentially allow autologous treatment in aesthetic and clinical tissues conditions in which FGF has a leading role. The versatility and broad beneficial effect of ozone has become evident in orthopedics, cutaneous and mucosal infections as well as in dentistry. The induction of FGF and other growth factors by ozone can support and potentiate those applications.*

Introduction

Application of basic fibroblast growth factor (bFGF) has been shown to promote cellular proliferation and collagen synthesis in vivo. In vitro and in vivo studies have shown that bFGF is both a powerful stimulator of angiogenesis and a regulator of cellular migration and proliferation, and is markedly up-regulated following tendon injury and active at multiple stages of the healing process [1]. Use of FGF as a therapeutic agent for the treatment of ischemic cardiovascular disease is promising and clinical trials are in progress. FGF has pleiotropic roles in many cell types and tissues: it is a mitogenic, angiogenic and survival factor involved in cell migration, cell differentiation and in a variety of developmental processes. Although devoid of signal peptide, FGF could be secreted. It acts mainly through a paracrine/autocrine mechanism involving high affinity transmembrane receptors and heparin sulphate proteoglycan low affinity receptors, but also through a still unknown intracrine process(es) on intracellular targets. FGF has many biological functions which are probably isoform-specific. bFGF may sensitively regulate local bone resorption and remodeling through direct and indirect mechanisms that promote angiogenesis and osteocyte recruitment, formation, differentiation and activated bone pit resorption [2]. It has been demonstrated that ozone (O_3) can promote platelet aggregation particularly when heparin is used as an anticoagulant. Platelet-rich plasma (PRP) treated with O_3 significantly increases the amount of platelet-derived growth factor (PDGF), transforming growth factor b1 (TGF-b 1) and interleukin-8 (IL-8). These factors are released in a dose-dependent manner after ozonation of heparinised PRP samples. These findings may partially explain the enhanced healing of topical ulcers in patients with chronic limb ischemia treated with O_3 autohaemoteraphy (O_3-AHT) [3]. FGF plays a role in various stages of development and morphogenesis, as well as in angiogenesis and wound healing processes. That is why the aim of this study was to evaluate the efficacy of O_3 to induce the release of FGF in PRP.

Materials and Methods

The O_2/O_3 mixture was generated just before application by an Alnitec Ozo2Futura device (Italy). Ozone obtained from medical grade oxygen represented about 0.4-0.5% of the gas mixture. The ozone concentration was measured using a built-in UV spectrophotometer at 254 nm.

Reagents

Anticoagulants were either heparin (calcium salt, 30 IU/mL blood) normally used for therapeutic purposes (Clarisco, Teofarma srl, Pavia. Italy) or ACD (citric acid, sodium citrate, glucose) (Haemonetics, Braintree, MA, USA).

Preparation of platelet-rich plasma samples

Serum, ACD or heparinised PRP were prepared from the same blood samples (18 mL) drawn from five fasting (12h) non-smoker volunteers between the ages of 23 and 60 years, who were considered to be healthy and had not ingested platelet-active medication for at least two weeks. This study was approved by an institutional review board (Scientific and Ethics Committees of the Institution) in accordance with the principle of the Declaration of Helsinki concerning the Ethical Principles for Medical Research Involving Human Subjects [4]. All volunteers signed an informed consent form before being enrolled. All patients were given adequate information (characteristics of the study, benefits and possible side-effects). Before enrolling, all participants attended a training programme to familiarize with the study objectives.

Nine parts blood were mixed with one part of saline or anticoagulated with either one part ACD or with one part of saline containing heparin so the final concentration was 30 IU/mL. Blood was centrifuged at 200 g for 20 min and platelets were measured with a Coulter counter. An average platelet count of 3 x 108/mL serum / plasma was used.

Treatment of biological samples

A volume of 0.4 mL of PPR was mixed with 25 µL saline, 25 µL of CaCl 10%, 25 µL of saline plus 0.4 mL of O_2/O_3 gas mixture (O_3 concentration of 80 µg/mL) or 25 µL of saline plus 0.4 mL of O_2. O_2/O_3 or O_2-treated samples were collected with a silicone coated disposable syringe and immediately introduced into a second syringe containing an equivalent volume of PRP via a 'y' connector. Final gas pressure remained at normal atmospheric pressure. The PRP samples were gently but continuously mixed with the gas for up to 30 s and afterwards the "y" were dispensed into test tubes for FGF analysis. After 2h incubation each sample was immediately centrifuged at 10 000 g for 20 min at 2°C and the supernatant platelet-free was frozen at –70°C until determination of FGF.

Immunoassay

Immunoassays of human bFGF were carried out using immunoassay kits produced by EMELCA Bioscience (Breda, Netherlands). On the basis of preliminary tests samples were diluted. A Bio-Rad 680 microplate reader (Hemel Hempstead, UK.) at 450 nm was used to read the samples absorbance. Samples were tested at least in duplicate against the appropriate standards.

Statistical analysis

The Outliers preliminary test for detection of error values was initially applied. Afterwards, data were analyzed for normality using the Shapiro-Wilk W test followed by homogeneity variance test (Levene). In addition, descriptive statistics was done. Results are expressed as the mean ± the standard deviation of the mean (S.D.). A software package was used for data collection and statistical analysis (Statistics for Windows 17.0.2, USA). The significance of the differences between the means in each group was analyzed by one-way analysis of variance (ANOVA). The level of statistical significance was set at $p<0.05$ for both inter and intra-groups analysis.

Results

The concentration of FGF was increased by approximately 580-700 percent after 2h incubation of PRP with saline, Ca2+, O_2 or O_2/O_3 compared to the basal concentration 1.636±0.969 pg/mL. Statistical differences were not detected among the concentrations of FGF from platelets collected in ACD, heparin or those obtained from serum. The intra group analysis (different treatment between the same samples) showed no significant differences in FGF concentration in samples of PRP treated with saline, Ca2+, O_2 or O_2/O_3 (Table 1).

Discussion

It has been demonstrated that exposure of PRP to O_2/O_3 stimulates the release of different growth factors3,5, but the effect of this procedure on the induction of FGF has not been reported. As demonstrated (Table 1) during O_2/O_3 exposure, PRP released similar quantities of FGF as when incubated with other substrates. FGF has an essential role in the differentiation of stem cells [6,7] and is a potent in vitro mitogen for capillary endothelial cells, stimulating angiogenesis in vivo, and may participate in tissue repair [2]. In contrast with other PRP treatments O_2/O_3 exposure releases significant ($p<0.05$) quantities of other platelet growth factors (Figure 1), and this pool of factors is thought to participate in the regeneration of tissues.

PRP is a rich source of growth factors and promoted significant changes in monocyte-mediated

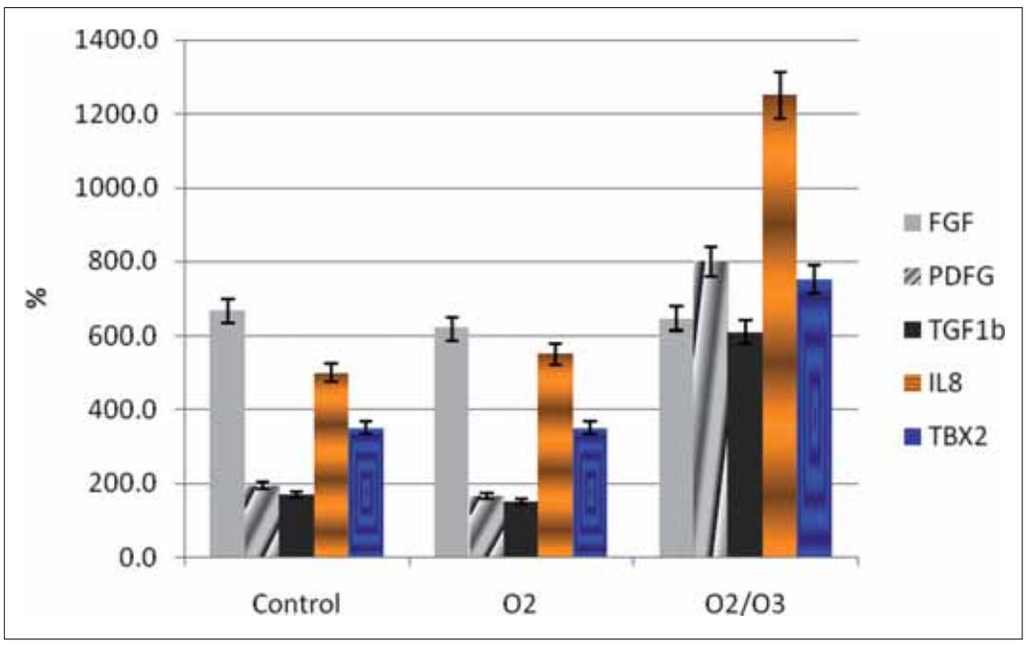

Figure 1 Shows that incubation of PRP collected in heparin, untreated (control) or exposed to O_2 or O_2/O_3 dramatically increased the release of platelet growth factors compared to the basal level. The highest and significant ($p<0.05$) increment was reached in the O_2/O_3 treated group for PDFG, TGF1b, IL8 and TBX2. However, the increment in the concentration of FGF has the same proportion in groups untreated or exposed to O_2 or O_2/O_3 samples.

proinflammatory cytokine/chemokine release. LXA4 was increased in PRP, suggesting that PRP may suppress cytokine release, limit inflammation, and, thereby, promote tissue regeneration [8]. Platelet activation allows access to autologous growth factors which by definition are neither toxic nor immunogenic and capable of accelerating the normal processes of bone regeneration. PRP can thus be considered a useful instrument for increasing the quality of regenerated bone [9], wound healing [10], healing of injury-associated soft tissue defects [11], chronic non-healing tendon injuries including lateral epicondylitis and plantar fasciitis and cartilage degeneration [12].

Ozone therapy has been used for many years as a method ancillary to basic treatment, especially in those cases in which traditional treatment methods do not give satisfactory results, e.g. skin loss in non-healing wounds, ulcers, pressure sores, and fistulae. Clinical results demonstrate the efficacy of this procedure in post-surgical and post-trauma complications [13], diabetic food [14], and others. Some recent studies also demonstrate that part of the wound repair mechanisms of ozone or ozone-derived products are associated with an increased expression of PDGF, TGF-beta, and VEGF [15].

As demonstrated, ozone does not modify the increment in the release of FGF from PRP. However, O_2/O_3 stimulates the release of a number of growth factors present in PRP, including PDGF, TGF-b1, IL-8 and TBX2. The ability of ozone to

Table 1 Release of bFGF from human platelets during 2h incubation. The PRP samples in saline, heparin or ACD were not exposed (control), or exposed to Ca2+, O_2 or O_2/O_3 (80 µg/mL) for 30 s before incubation.

Sample		*NaCl*	*O_2/O_3 80 µg/mL*	*O_2*	*Ca2+*
Plasma collected in heparin	Mean S.D. % I	10.84 1.49 662	10.50 1.40 642	10.07 1.26 616	11.21 1.89 685
Plasma collected in ACD	Mean S.D. % I	11.61 3.32 709	9.83 2.37 601	9.62 2.33 588	10.61 2.83 648
Serum	Mean S.D. % I	9.86 2.61 602	9.50 1.43 580	9.58 2.41 586	10.96 2.89 670

stimulate the release of other important growth factors from platelets, like vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF), should be tested. In the early stages of wound healing, platelets are activated by thrombin and collagen, and growth factors are released by these activated platelets to facilitate repair and healing. The sequence of events leading to bone formation and wound healing (chemotaxis, cell migration, proliferation, and differentiation) [16] are regulated by growth factors [17], many of which are present in PRP. For example, the recruitment of mesenchymal stem cells and progenitor cells to the site of bone regeneration is mediated by collagen as well as chemotactic factors such as PDGF and TGF-b1. Moreover, PDGF and TGF-b1 stimulate cell proliferation [18], and TGF-b1 also induces the osteogenic differentiation of mesenchymal stem cells [19]. Osteoblast differentiation is controlled by IGFs and bone morphogenetic proteins [16], and VEGF is critical in stimulating the angiogenesis necessary for bone formation and remodeling.

Conclusions

The compelling advantages of PRP for bone repair and wound healing and the capacity of ozone to stimulate the release of growth factors is an important point for future research on this topic. Future studies will also focus on the optimization of release of growth factors. The objective is to tailor the release cascades to match those required for tissue regeneration, enabling essential factors to be available at physiologically effective concentrations and during critical periods of remodelling.

Basal concentration of bFGF in non-treated serum/plasma was 1.636 ± 0.969 pg/mL. The mean platelet concentration used was $447.8 \cdot 10^3/\mu L$. ACD, citric acid, sodium citrate, glucose. PPR, platelet-rich plasma. I% increment in bFGF compared to basal bFGF concentration. Non-statistical differences were detected when compared to the concentration of FGF among groups. Statistical differences ($p<0.001$) were detected between basal concentration and post incubation samples in all cases.

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