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The effect of Platelet Lysate on osteoblast proliferation associated with a transient increase of the inflammatory response in bone regeneration

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ABSTRACT

Platelet Lysate (PL) contains a cocktail of growth factors and cytokines, which actively participates in tissue repair and its clinical application has been broadly described. The aim of this study was to assess the regenerative potential of PL for bone repair. We demonstrated that PL stimulation induces a transient increase of the inflammatory response in quiescent human osteoblasts, via NF-kB activation, COX-2 induction, PGE_2 production and secretion of pro-inflammatory cytokines. Furthermore, we showed that long-term PL stimulation enhances proliferation of actively replicating osteoblasts, without affecting their differentiation potential, along with changes of cell morphology, resulting in increased cell density at confluence. In confluent resting osteoblasts, PL treatment induced resumption of proliferation, change in cell morphology and increase of cell density at confluence. A burst of PL treatment (24-h) was sufficient to trigger such processes in both conditions. These results correlated with up-regulation of the proliferative and survival pathways ERKs and Akt and with cell cycle re-activation via induction of CyclinD1 and phosphorylation of Rb, following PL stimulation. Our findings demonstrate that PL treatment results in activation and expansion of resting osteoblasts, without affecting their differentiation potential. Therefore PL represents a good therapeutic candidate in regenerative medicine for bone repair.

1. Introduction

Large bone defects (critical-size defects) caused by disease, trauma or tumour resection are not healed by the intrinsic regenerative capacity of our body and remain one of the more difficult repair therapies to successfully achieve.

In the last years, to mimic and lead bone tissue regeneration, several methods involving novel biomaterials and different bone progenitor cell culture systems have been implemented that represented a step forward on the bone reparation road [1–9]. However, the other side of the coin is that cell-biomaterial implants present a list of disadvantages, such as the requirement of the cell culture in a strictly controlled environment, the high costs the need of optimized fully-resorbable scaffolds and the vascularization supply of large implants.

In light of these considerations, although tissue engineering for bone repair is certainly a feasible procedure, it might represent a suitable approach in case of extreme pathological conditions, whereas for most patients a non-surgical repair induction could be a much better and clinically feasible solution.

The healing process represents the physiological adaptation of an injured tissue, aiming at fully restoring its structural and functional integrity. When a tissue undergo a lesion, the recovery process begins with clot formation and platelet degranulation at the site of the injury, promoting the release of chemical signals involved in all aspect of the wound healing process and possibly triggering and regulating this process in a process which is similar for all tissue types [10,11].

Inflammation, the first phase of the healing process, leads the repair of the damage in the end, although it can also represent the main cause of tissue degeneration if it lasts for too long or becomes a systemic inflammation [12]. Thus, the modulation of the inflammatory response is a key factor to orchestrate the tissue repair.

During the inflammatory phase, the formed clot releases several platelet-derived chemokines and cytokines. In the last years,

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platelet derivatives known as Platelet-Rich Plasma (PRP) and Platelet Lysate (PL) have attracted the attention of several investigators. PRP and PL contain a cocktail of growth factors, cytokines and molecules that actively participates in the tissue repairing process. In light of their properties, these platelet by-products in the form of PRP and PL have been extensively used for clinical applications such as musculoskeletal injuries [10], wounds and soft tissue injuries [13], sports medicine [14–16], orthopaedics [17–22], dentistry [23–25], dermatology [26,27], ophthalmology [28–31], plastic [32] and maxillofacial surgery [33–36], soft tissues damage repair [37,38] and even as fetal calf serum substitutes for in vitro cell culture [39].

In this study we considered and attempted to investigate in culture some of the physiological events occurring during the initial phases of the wound healing process (i.e. blood extravasation, coagulation, platelet content release, stimulation of resident cells) eventually resulting in new tissue and repair.

Since our specific interest was directed to the bone fracture, we focused our work on the in vitro analysis of primary osteoblast cultures.

Osteoblasts play a pivotal role during bone formation and remodelling; mesenchymal stem cells can get activated and differentiate into osteoblasts precursors, which in turn, under the appropriate local stimulation, give rise to more functionally mature osteoblasts releasing extracellular matrix proteins and factors and regulating bone deposition and mineralization [40–43]. During the inflammatory phase of the healing process, bone morphogenetic proteins, produced by osteoprogenitor cells, together with inflammation cytokines, recruit mesenchymal cells and push their differentiation into more committed cells [44–46].

Our study started on the observation that a large amount of actively proliferating cells migrated out of bone chips treated with PL in vitro, compared to control cultures maintained in unsupplemented conditions. Our intent was to understand the putative regenerative effect of platelet-released molecules when a bone insult occurs and to elucidate part of the cellular and biochemical cascades of events involving the osteoblast population, trying to separate and to identify the single steps and the activated molecular pathways.

Considering that the healing process begins with blood extravasation, clotting and onset of inflammation, we first performed a biochemical analysis of the effect of the platelet content on the osteoblasts, both in physiological and inflammatory conditions, in terms of their proteins expression, release of active biological factors and pathways activation. Subsequently, we investigated the possible mitotic effect of PL on osteoblast proliferation, in presence and absence of inflammation, together with the maintenance of the differentiation potential of the treated cells. As well, we performed a set of experiments aiming at clarifying the effectiveness of PL in inducing proliferation, after a treatment limited in time, in line with what physiologically happens during the fracture inflammatory phase. Finally we investigated the possible actions of PL on the activation of proliferation pathways and the expression of specific proteins controlling the cell cycle.

2. Materials and methods

2.1. Materials

Iscove medium (Iscove MEM), L-glutamine, trypsin/EDTA, and penicillin/streptomycin were all obtained from Euroclone Life Sciences Division (Milan, Italy). Fetal calf serum (FCS), collagenase I, and collagenase II were purchased from Gibco (Invitrogen s.r.l, Carlsbad, CA). Ascorbic acid, dexamethasone, β-glycerophosphate, BAY 11-7082, UO126, LY294002, tyazolyl blue (MTT), Alkaline Phosphatase staining kit, Alizarin Red S, TRIreagent® and absolute ethanol were purchased from Sigma–Aldrich (St. Louis, MO.). Dispase II was purchased from Roche Applied Science (MB, Italy). Antibodies anti IL-6, anti IL-8, anti Actin and anti CyclinD1 were from Santa Cruz Biotechnology Inc. (Dallas, TX). Antibodies anti COX-2, anti microsomal Prostaglandin E Synthase-1 (mPGES) anti haematopoietic Prostaglandin D Synthase (hPGDS) were from Cayman Chemical (Ann Arbor, MI). Antibody anti phospho-Rb was from Cell Signalling Technology (Danvers, MA).

2.2. Preparation of purified Platelet Lysate

Platelet Lysate was prepared according to Doucet et al. and Zaky et al. [39,47] with little modifications. In brief, platelet rich plasma (PRP) was obtained from healthy human blood donors from the San Martino Hospital Blood Transfusion Centre (Genoa, Italy) in consensus with the guidelines of the institutional ethics committee. After a low speed centrifugation, platelets were re-suspended in physiologic saline (0.9% NaCl, Fresenius Kabi, Italy) to eliminate possible contaminants from plasma; the platelet concentration was adjusted to 1×10^7 platelets/μl in PBS and the resulting suspension subjected to 3 consecutive freeze–thaw cycles to obtain platelet activation and lysis. In order to precipitate broken platelet membranes, a high-speed centrifugation was performed and the supernatant, containing the cocktail of factors released by the platelets re-suspended in PBS (Platelet Lysate, PL), was collected and stored in aliquots at −80 °C until use. To minimize variations between donors, PL preparations used for this study were derived from pooled PRP preparations deriving from at least 10 donors.

2.3. Chips culture and osteoblast isolation

Samples of human bone tissue were obtained from trabecular bone removed from the femoral shaft of adult patients that underwent hip joint replacement surgery at the San Martino Hospital Orthopaedic Clinic (Genoa, Italy). The informed consensus of the patients and the approval of the institutional ethics committee were preliminary obtained.

For chips culture, trabecular bone fragments were cultured in complete medium (Iscove medium containing 10% FCS) either additionally supplemented or not with 5% PL. After twelve days when outgrowth of adherent cells from the tissue explant was observed, bone chips were removed, washed with PBS and further cultured in complete medium without PL for additional six days. Primary osteoblasts were isolated from 48 femoral heads derived from patients experiencing fracture of the femur or with a severe osteoarthritis. The patient age range was 64–85 years. Only two patients were 41 and 54 years old respectively.

Bone samples were cleaned of adherent soft tissues and cut into small pieces (2 mm × 2 mm). After cutting, samples were washed in PBS and digested firstly in 1 mg/ml trypsin/Ringer solution (147 mM NaCl, 4 mM KCl, 1.13 mM $CaCl_2$ in water) for 10 min at 37 °C, then in 2 mg/ml dispase/Ringer solution for 20 min at 37 °C and finally in 3 mg/ml collagenase I/Ringer solution twice for 30 min at 37 °C. Cells released by the collagenase digestion were washed twice in PBS and grown to confluence in Iscove medium supplemented with 2 mM glutamine and 50 mg/ml penicillin/streptomycin and 10% FCS (complete medium). Cells were maintained in a controlled environment at 37 °C in a humidify atmosphere of 5% CO_2 and 95% air. The medium was initially changed 24 h after the procedure and then every 2–3 days. All experiments were performed using cells cultured for no longer than 2 passages.

2.4. Cells stimulation by Platelet Lysate and IL-1α

The in vitro experiments were performed to mimic the in vivo effect of the mixture of platelet-derived factors, physiologically released at the wound site, on resident osteoblasts.

For long-term stimulation experiments, the osteoblast complete medium (Iscove medium with 10% FCS) was supplemented with one of the following compounds: 1) no supplements added (control condition); 2) 5% PL; 3) 100 U/ml IL-1α; 4) PL + IL-1α. Culture medium was changed every 3 days.

For experiments in which the stimulation was performed only in a time-limited approach, cells were stimulated with the medium enriched with PL and/or IL-1α only for the initial 24 h. After this "burst" of treatment cells were maintained in control complete medium (no supplements other than 10% FCS).

2.5. Osteogenic differentiation

To induce osteogenic differentiation, confluent osteoblasts were cultured in differentiation medium (DM): Iscove medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μg/ml penicillin/streptomycin, 50 μg/ml ascorbic acid, 1.5 mg/ml β-glycerophosphate, and 10^{-7} M dexamethasone [5]. PL, IL-1α or a combination of the two was added on the basis of the required treatment. The osteogenic differentiation treatment lasted for 21 days. Alizarin red and alkaline phosphatase staining were performed at different time points following the manufacturer's instructions.

2.6. Proliferation assay

To evaluate cell viability and proliferation, the tyazolyl blue (MTT) method was used. The culture medium was removed and replaced with 1 ml of serum-free Iscove medium supplemented with 50 μl of MTT stock solution (5 mg/ml). After 3-h incubation, the medium was discarded and the converted dye was solubilized from the