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PEGylated liposomes encapsulating human hemoglobin enhance oxygen transfer and cell proliferation while decreasing cell hypoxia in fibrin

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ABSTRACT

Oxygen supply to cells in three-dimensional cultures occurs mainly by diffusion, with insufficient oxygen concentration reaching the center of cell aggregates, thus creating hypoxic areas and leading eventually to cell death. The aims of this study were, firstly to measure and characterize the oxygen transfer from liposomes encapsulating human hemoglobin (LEH) to an aqueous environment and, secondly, to study the effects of LEH to cells grown in a three-dimensional environment. LEH were loaded with pure oxygen and the oxygen transfer from the liposomes to an aqueous phase was monitored. Human umbilical vein endothelial cells (HUVEC) were cultured in a fibrin gel mimicking a diffusion-limited environment. After 24 h, HUVEC seeded in fibrin covered with culture medium supplemented with oxygen-loaded LEH showed a decrease in HIF-1α expression and an increase in proliferation compared to those in fibrin cultured either with medium containing liposomes with no hemoglobin or non-modified medium.

1. Introduction

In the growth of tissue mass, the available concentration of oxygen to cells is often a limiting factor [1,2]. The absence of a microvessel network to perfuse nutrients and oxygen at the initial stage of a tissue culture eventually leads to the necrosis of the growing tissue. The slow diffusion through culture medium and tissue mass limits oxygen transport, a major obstacle in achieving desirable cell density and cell functionality [3,4]. This is particularly true for *in vitro* cultures, where cells are located far from an oxygen source. One of the main problems encountered is to bring sufficient oxygen to cells located in the core of aggregates [2], inevitably leading to oxygen-deprived regions (i.e., hypoxic zones) and thus limiting the thickness of the viable tissue and normal tissue growth.

In a tissue or organ, hypoxia can be defined as an oxygen concentration below "normal" physiological range [5–8]. Often it is difficult to distinguish normal and abnormal limits. The oxygen concentration available to cells depends on the type of tissues, with possible variation among a given tissue or organ [9–12]. Hypoxic conditions are found during embryogenesis, as hypoxia is involved in normal development [13,14]. But on the other hand, hypoxia can cause deleterious effects to cells [15,16], and these effects on cell and tissue development and function seems to depend on its intensity. For example, hypoxia can support cell proliferation and survival [7,17–20], while it can result to cell apoptosis [21–23]. A delicate balance must be reached between the stimulatory and inhibitory effects of low oxygen supply on cell responses.

Fibrin gels are formed by the reaction between fibrinogen and thrombin. Their composition, physicochemical properties and uses have been well characterized in the scientific literature [24]. *In vivo*, following vascular injury, fibrinogen is cleaved by thrombin to form insoluble fibrin clots. The clots serve as temporary matrices for cell adhesion and migration during tissue repair [25]. Fibrin gels can also serve as three-dimensional substrates in which cells are seeded and allowed to proliferate [25]. The ability to modulate the thickness of the gel provides a good way to investigate the effect of diffusion limitation and hypoxia on cells seeded at different levels in the matrix.

Hypoxic conditions often cannot be avoided due to the longer diffusion time of oxygen [26]. With this in mind, we have recreated a three-dimensional environment similar to culture conditions in which cells in the core of aggregates or scaffolds do not have direct access to nutrients and oxygen [2].

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To increase oxygen supply to cells, oxygen carriers such as perfluorocarbon-based or hemoglobin-based systems have been considered [2,27]. For oxygen carriers to be used in a tissue culture, they should be able to deliver closely and effectively higher oxygen concentrations than those obtained with dissolved oxygen alone. Many papers have been published about these types of carriers [2,28–37]. In this study, liposomes encapsulating hemoglobin (LEH) have been selected and considered as possible oxygen reservoirs [2,38].

The use of oxygen carriers, as blood replacement, has been assessed *in vivo* [39–42]. In the near past, several products promoted as blood replacement therapy have failed in clinical trials due, in part, to their toxicity [2,28,43]. Cytotoxicity concerns have also been brought up in the *in vitro* use of these oxygen carriers [37]. Harmful side-products can be released and cause deleterious effects on cells.

Cytotoxicity of LEH depends greatly on the composition of the liposome formulation. In a previous study, exposing endothelial cell monolayers to LEH revealed that the LEH formulation was cytotoxic [37]. Knowing the toxicity involved with direct exposure of this LEH composition to cell monolayers, we hypothesized that LEH cytotoxicity could be decreased (even eliminated) if these carriers were used to supply oxygen to cells dispersed in a three-dimensional matrix and eventually circulated through this matrix. In this case, cells would not be in direct contact with LEH or time of direct exposure would be reduced.

The first objective of this paper was to investigate the oxygen transfer from liposomes encapsulating functional human hemoglobin to an aqueous environment. Secondly, we investigated whether LEH could transport and transfer sufficient oxygen to human endothelial cells grown in a three-dimensional *in vitro* environment. To confirm this second goal, cell hypoxia was studied to compare the state of cells supplemented with liposomes containing oxygen-loaded hemoglobin to that of cells cultured without LEH or with empty liposomes (i.e., with no hemoglobin).

2. Materials and methods

2.1. Reagents

The following reagents were purchased from Sigma–Aldrich (Oakville, ON, Canada): bovine serum albumin (BSA), endothelial cell growth supplement (ECGS), foetal bovine serum (FBS), fibrinogen, goat serum, glycin, Hank's balanced salt solution (HBSS), heparin, Medium 199 (M-199), plasmin and Triton X-100.

Paraformaldehyde, phosphate buffer solution (PBS) and Tween-20 were obtained from Fisher Scientific (Ottawa, Ontario, Canada). Antibiotics for cell culture, trypsin and Prolong Gold Antifade Mounting Medium were purchased from Invitrogen (Burlington, ON, Canada). Thrombin was obtained from Calbiochem (San Diego, CA, USA). Fish gelatin was a gift from Gilles Grondin (Département de Biologie, Université de Sherbrooke, Sherbrooke, Canada).

2.2. Liposome preparation

PEGylated liposomes containing human hemoglobin were prepared and characterized as previously described [37]. In brief, liposomes were made of a mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), cholesterol, palmitic acid and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000] in a molar ratio of 5/5/1/(0.3%[mole/total moles of lipids]). In the final step, the hydrated lipid mixture, prepared as previously described [37], was dispersed into a hemoglobin solution (0.4 g/mL) and the resulting multilamellar vesicles were extruded with the LiposoFast-Basic (Avestin Inc., Ottawa, ON, Canada) through polycarbonate membranes with pore sizes of 200 nm, as also previously reported [37]. The resulting suspension was then ultra-centrifuged at 50,000 × *g* for 30 min (Optima TLX, Beckman Coulter, Mississauga, ON) to recover and eliminate the top layer containing non-encapsulated hemoglobin. Hemoglobin used in this study was obtained from whole blood of healthy volunteers. All procedures were approved by the Ethics Committee of the Research Centre on Aging (Sherbrooke, Qc). After the subjects were thoroughly informed about the nature and goal of the study, they provided written consent. Purification and characterization were achieved using previously published methods and results are briefly recalled ([Hb] = 30 g/dL; [metHb] ≤ 2%; pH at 37 °C = 7.4) [37,44,45]. Pyridoxal 5′-phosphate (PLP) (18 mM) was also encapsulated with hemoglobin to yield an estimated P_{50} of 30 Torr [44,46,47]. Hemoglobin function to load and unload oxygen was assessed using visible-light spectroscopy, as previously described [37]. Empty liposomes were prepared in the same way, but using M-199 media in substitution for hemoglobin solution.

2.3. Culture of human endothelial cells in monolayers

Human Umbilical Vein Endothelial Cells (HUVEC, PromoCell, Heidelberg, Germany) were cultured in M-199 supplemented with 10% (v/v) de-complemented FBS. ECGS (20 μg/mL), heparin (90 mg/L), and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) were also added. Culture media were replenished three times a week. All cells were maintained at 37 °C in an incubator with a humidified atmosphere containing 5% CO_2. HUVEC from passages 4 to 6 were used throughout this study. When cells reached confluence, they were trypsinized, counted and re-suspended to attain a concentration of 1 million cells/mL in M-199 with no heparin.

2.4. Fibrin containing HUVEC

HUVEC were entrapped into fibrin by mixing equal volumes (400 μL total) of thrombin (1.75 U/mL, in HBSS buffer) and fibrinogen (1.75 mg/mL, in HBSS buffer) in 48-well plates. All solutions were pre-heated at 37 °C before use. 200 μL of a fibrinogen solution was placed into a well. Cells (250,000 cells/mL) were added and mixed using an automatic pipette. Immediately after mixing the cell-fibrinogen suspension, 200 μL of thrombin solution was added drop-by-drop, but as quickly as possible (200 μL were added drop-by-drop in less than 5 s), to the solution already containing fibrinogen and cells. It is important to note that if thrombin is added quickly in one shot and not drop-by-drop, "web-like" structures could form and make microscopic observations difficult. The mixture was incubated for 5 min at 37 °C to allow polymerization. Afterwards, M-199 medium containing 10% FBS and heparin was added. Cells were allowed to grow and differentiate for 24 h. After 24 h, culture medium was aspirated and replaced with either: (1) PEGylated liposomes containing human hemoglobin, (2) empty liposomes or (3) M-199, all of which contained culture media and 10% FBS.

2.5. Oxygen-loading procedure

Hemoglobin is known to possess characteristic bands throughout the visible spectra. The Q-band (around 500–600 nm) and Soret band (in the blue region, around 400 nm) are typical regions that provide information about the hemoglobin state [37,48]. Functionality of hemoglobin and its function to shift from its oxygen-loaded position to unloaded position can be assayed by visible-light spectroscopy [2].