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Cytoskeleton-specific immunoliposomes: sealing of hypoxic cells and intracellular delivery of DNA

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Abstract

Various pathological conditions, including hypoxia and inflammation, provoke cell membrane lesions. These lesions represent microscopic holes in the sarcolemma through which the components of the cytoskeleton become exposed to the surroundings. Labeled antibodies against intracellular cytoskeletal antigens, such as antimyosin antibody, may be used to reveal cell membrane lesions. Being coupled to liposomes, such antibodies can deliver phospholipid vesicles to the affected cell surface and 'plug' them directly into the holes. The in vitro antimyosin antibody-mediated liposome transport to cytoskeletal antigen of hypoxic cardiomyocytes was used in order: (1) to prevent cell deaths by sealing membrane lesions; and (2) to achieve intracellular DNA delivery. A hypoxic model of injury in H9C2 rat embryonic cardiocytes was used in these experiments. Under hypoxic culture conditions, cells were incubated with 150–200 nm antimyosin-immunoliposomes (IL), plain liposomes (PL), and non-specific IgG-liposomes (IgL). After hypoxia (which lasted in different experiments from 1 to 5 days), cell viability was assessed following [^{3}H]thymidine incorporation, Trypan Blue exclusion test and by fluorescent microscopy. All tests demonstrated highly improved survival of hypoxic cells in the presence of IL (up to 95% survival after 24 h of hypoxia), whereas, cell survival in the presence of control PL and IgL never exceeded 40%. The presence of IL maintained the survival of hypoxic cells for approximately 5 days, while all control cultures never survived for more than 24–36 h. In addition, salvaged cells maintained normal proliferation after hypoxia in the presence of IL. Electron microscopy experiments with silver grains containing IL demonstrated that after incubation, silver grains can be identified in the cytoplasm, which we see as evidence of possible fusion of 'plugging' liposomes with cell membrane. Based on this phenomenon, it is hypothesized that if target cells are under artificial stress, cytoskeleton-specific IL can deliver their contents into the cytoplasm, which provides a good opportunity for the intracellular delivery of drugs and DNA. This hypothesis was confirmed by successful delivery of a plasmid pEScFv 2G42D7 (antimyosin antibody) vector in hypoxic H9C2 cardiocytes by IL.

Keywords: Immunoliposomes; Cytoskeletal antigens; Cardiocytes; Hypoxia; Cell salvage; DNA delivery

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1. Introduction

Various pathological conditions including hypoxia and inflammation provoke cell membrane lesions. The presence of those lesions which represent microscopic holes in the sarcolemma permits washout of intracellular macromolecules into the circulation. In contrast, certain intracellular proteins, including the components of cytoskeleton (myosin, vimentin) become exposed through these holes to the surroundings. Appropriately labeled antibodies against intracellular cytoskeletal antigens may be used to reveal cell membrane lesions. Moreover, being coupled to liposomes, such antibodies acquire an ability to deliver phospholipid vesicles to the affected cell surface and anchor (plug) them directly into (over) the holes.

We suggested and experimentally tried to use antibody-directed liposome transport to cytoskeletal antigens to approach two problems which have important implications in medicine: (1) to prevent hypoxia-caused release of intracellular contents and subsequent cell death by sealing (plugging) membrane lesions with such immunoliposomes (IL), and (2) to use IL for intracellular delivery of pharmacologically important substances, such as drugs or genetic material.

2. Targeted sealing of cell membrane lesions: model of preservation of cell viability by immunoliposome therapy

The hallmark of necrotic cell death is the loss of cell membrane integrity as evidenced by the presence of cell membrane lesions. In acute myocardial infarction, for example, membrane lesions have been documented by ultrastructural studies with as little as 20 min of ischaemia (Jennings et al., 1985). Antimyosin antibody, a marker of an intracellular cytoskeletal antigen, has been used to demonstrate sacrolemmal lesions indicative of necrotic myocyte damage (Khaw et al., 1976, 1979). Hypoxia-provoked membrane disruption can not be reversed by simple restoration of blood flow to the myocardium at risk. Moreover, reperfusion may even accelerate irreversible cell death by greater washout of intracellular contents, reperfusion injury and explosive cell swelling (Reimer et al., 1983; Braunwald and Kloner, 1985).

To minimize cell death and preserve the viability of compromised myocardium, we hypothesized that if the membrane lesions could be sealed to prevent loss of intracellular contents, then the treated cells should recover and remain viable. Cells under ischemic (or hypoxic) conditions will initially develop small cell membrane lesions which would enlarge with extended period of ischaemia. Even at early time points of hypoxia, intracellular contents may leak out, spear-headed by ions followed by larger macromolecules leading to irreversible cell death. If the lesions can be plugged early enough and normoxia restored, the treated cells should remain viable. To achieve this, the use of antibody-targeted liposome-plugs is suggested (Khaw et al., 1995). A cytoskeletal antigen exposed via membrane lesion can be used to anchor the immunoliposome-plug to provide the initial seal and prevent washout of the intracellular macromolecules (see graphic representation of our hypothesis in Fig. 1).

The phenomenon of plug and seal to prevent necrotic cell death was demonstrated using myosin as the cytoskeletal target antigen and the corresponding antimyosin antibody as the anchoring device incorporated in liposomes in a hypoxic model of injury in H9C2 rat embryonic

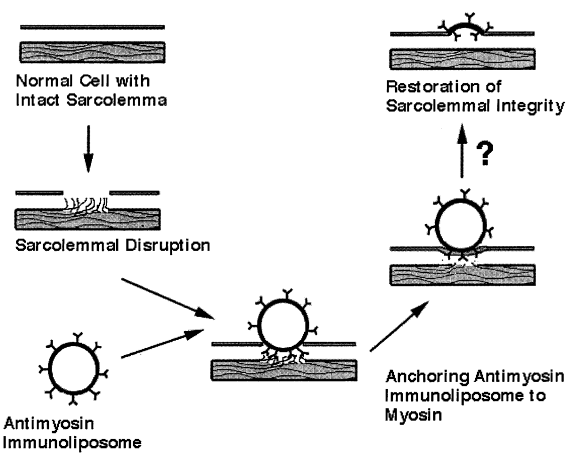

Fig. 1. Diagrammatic representation of the hypothesized mechanism of cell membrane sealing and salvage by antimyosin liposomes. Adapted from Khaw et al. (1995).

Table 1
Assessment of cell viability by Trypan Blue exclusion test and by following 3HT uptake

Cells and treatment conditions	Viability by Trypan Blue (% of initial cell number)	Viability by 3HT (% of control)
NC (control)	98.30 ± 0.58	100
HC	13.97 ± 1.77	3.21 ± 4.22
HC+PL	42.30 ± 3.11	30.99 ± 15.7
HC+IgL	42.85 ± 6.24	—
HC+IL	96.17 ± 1.24	88.59 ± 13.02

Viability by Trypan Blue was assessed utilizing all cells in each culture flask in triplicates ($n = 3$, per group). Viability by 3HT uptake was assessed by incubating myocytes cultured under hypoxic or normoxic conditions for 24 h with or without liposomes with 5 μCi of 3HT under normal culture conditions for another 24 h ($n = 6$, per group).

cardiocytes (Khaw et al., 1995). For this purpose, 2 million H9C2 cardiocytes were incubated with 10% FCS in DMEM at 37°C, 5% CO_2. After overnight incubation, the cells were washed in phosphate-buffered saline and recultured in a fresh medium (with or without liposomes). Nitrogen was bubbled through the medium vigorously for 4 min dislodging all cells ($> 95\%$) from the bottom of the flasks. The flasks were then closed tightly to maintain hypoxia through overnight incubation at 37°C.

H9C2 cardiocytes in hypoxic culture conditions were incubated with antimyosin-IL, plain liposomes (PL) and control non-specific IgG liposomes (IgL). Hypoxic and normoxic cardiocytes (NC) without liposome treatment were used as additional controls.

Liposomes were prepared from a mixture of egg phosphatidylcholine and cholesterol at a molar ratio of 1:1. For incorporation into liposomes (when necessary), antimyosin antibody 2G42D7 was modified with *N*-glutaryl phosphatidyl ethanolamine (NGPE) as in (Weissig et al., 1986; Torchilin et al., 1992). The solution of NGPE-modified antibody was added to the detergent-solubilized lipids. Certain liposome preparations contained also fluorescent rhodamin-labeled lipids. The mixture was dialyzed overnight against PBS (pH 7.4) to remove the detergent. The resulting liposomes were extruded serially through 0.6, 0.4 and 0.2 μm pore size Nuclepore filters. Final liposome size was within 150–200 nm diameter.

Assessment of viability of the cells was performed after 24 h of hypoxia by Trypan Blue exclusion method or by immediate further incubation of the cells with [^{3}H]thymidine (3HT). According to Trypan Blue exclusion data, almost all control hypoxic cardiocytes (HC) were non-viable (Table 1). PL provided certain protection from hypoxic injury, probably by nonspecifically sticking to cell surfaces and fortuitously 'sea' some of the cell membrane breaches. IL almost completely prevented cell death with a cell viability similar to that of normoxic cells. Hypoxic cells treated with non-specific IgG-liposomes (IgL) demonstrated viability on the level of PL-treated cells.

3HT uptake studies indicative of DNA replication and cell viability, demonstrated similar patterns (Table 1). If mean 3HT uptake in control NC was assigned 100%, uptake in all hypoxic controls was only approximately 3%. Uptake in IL-treated hypoxic cells was almost 90% of normoxic cells, but significantly greater than in PL- or IgL-treated hypoxic controls ($\approx$ 30%).

Additional data confirming increased survival of hypoxic cells in the presence of IL were obtained by us in microscopy studies with the use of fluorescent liposomes (Khaw et al., 1995). It was found that HC treated with rhodamine-labeled PL had minimum fluorescence, were no longer confluent and only some cells remained attached to the flasks as small clusters of aggregated cells. Furthermore, isolated scattered adherent cells with abnormal cell shape were observed by confocal microscopy. Most of the PL-treated hypoxic cells were recovered in the supernatant as non-viable cells. On the other hand, HC treated with rhodamine-labeled IL had enhanced fluorescence

(i.e. increased liposome binding) and showed localization of fluorescent IL on the cell surfaces with near-normal-shaped cells by confocal microscopy. The cultures remained confluent and retained normal cell morphology.

Cell salvage may be due to fusion of the IL lipid bilayer with the cell membrane promoted by the anchoring of the IL-plug to the underlying cytoskeletal myofilaments with antimyosin on IL surface. The mechanism of cell salvage may also be by simple plugging of the sarcolemmal lesions with the IL kept in place by the antibody anchors (Fig. 1). Whatever the exact mechanism, this model of intervention provided a novel approach to preservation of cell viability which must be substantiated in subsequent in vivo studies.

IL treated hypoxic cells have survived more than 7 days in subsequent normoxic culture conditions and were observed to be replicating normally. Prevention of cell death by cell membrane lesion sealing as described could have significant clinical utility. Thus, for example, the availability of an effective therapeutic intervention for myocardial preservation following thrombolytic therapy in acute myocardial infarction with IL should provide incremental patient benefit.

It is, however, very important to determine how long this protective effect can last. Therefore we undertook a study to determine whether antimyosin IL can protect severely injured cardiocytes cultured under hypoxic conditions for 1–5 days (Khaw et al., 1996a). The experiment was designed as already described, the only difference being time periods during which cells were kept under hypoxic conditions. Cell viability was assessed following cell replication and by 3HT uptake. Untreated HC, NC, and cardiocytes treated with plain PL were used as controls.

Survival of NC as assessed by 3HT uptake, increased from 100% (the mean 3HT uptake in control NC was assigned 100%) to $253.8 \pm 15.7\%$ after 24 h (cell replication proceeds), whereas, virtually no survival of HC was registered after the same time period. PL added to HC provided certain viability after 24 h ($\approx 78\%$), which dropped to approximately 4% after 2 days of hypoxia, and to less than 1% after 3 and more days. However, when hypoxic cells were treated with IL, not only did IL confer protection, IL also permitted cell replication. This is evidenced by the similarity in the increase of 3HT uptake after 24 h in NC (253.8%) and in IL-treated HC ($224.8 \pm 13.02\%$). After 48 h or more of hypoxia the replication in IL-treated HC was decreased, but the viability of cells was maintained on the level of 48 ± 7.5, 14.8 ± 7.7 and $7.7 \pm 4.1\%$ after 3, 4 and 5 days of hypoxia, respectively. These data show that the protecting effect imposed by IL on hypoxic cells leads to a long-term preservation of cardiocyte viability which might be especially important from the practical point of view (long-term preservation of myocardial viability upon infarction and reperfusion).

3. Targeted intracellular delivery of drugs and genes by cytoskeleton-specific immunoliposomes

In an attempt to learn more about the exact mechanism of IL interaction with HC, we have performed certain ultrastructural studies using electron microscopy with silver-loaded electron-dense liposomes (Khaw et al., 1995). To prepare such liposomes (Torchilin et al., 1988), silver nitrate was added in a buffer solution during liposome preparation by sonication. Liposomes, purified from the non-entrapped silver nitrate by dialysis were supplemented with 0.12 M NaCl and exposed to the light for 1 h, which led to formation of fine electron-dense precipitate of silver oxide inside liposomes.

Electron microscopy of hypoxic cells incubated with silver oxide-loaded IL demonstrated internalization of the silver grains and their localization in the cytoplasmic compartment, denoting possible fusion of the IL with the cell membrane and release of the intraliposomal contents into the cytoplasm (Torchilin et al., 1988). This finding led to a conclusion, that artificially imposed hypoxia may be used to facilitate intracellular delivery of various drugs including DNA by cytoskeleton-specific IL.

It is hypothesized that if the target cells for drug and/or gene delivery are under naturally or artificially imposed stress, stress-induced small membrane lesions will allow intracellular traffick-

ing of liposomes rendered specific for an intracellular antigen. Such liposomes may simultaneously plug and seal stress-induced cell membrane lesions and provide intracellular drug (gene) release and delivery. This new technology, the possibility of which was demonstrated in our experiments with silver oxide-loaded antimyosin IL (Vural et al., 1996), was proved further by enhanced transfection and expression of a plasmid pEScFv 2G42D7 vector in H9C2 embryonic cardiocytes (Khaw et al., 1996b).

Plasmid pEScFv is an eucariotic expression vector containing antimyosin single chain Fv (ScFv) fragment linked with domain B of protein A of *Staphylococcus aureus*. Sp2/O myeloma cells transfected with pEScFv 2G42D7 secreted ScFv into the culture media. *E. coli* strain XL1/Blue was transformed with pEScFv 2G42D7 plasmid and plasmid DNA was obtained by alkaline lysis method (Sambrook et al., 1989) from the ampicillin-resistant line. Purified plasmid was checked by agarose electrophoresis.

Liposomes were obtained as described above with the only addition that the plasmid (50 μg/ml) was added to the lipid film together with NGPE-antibody and detergent. H9C2 cells were subjected to hypoxic stress as described and were kept under hypoxia for 6 h (mild hypoxia). After that, cells were washed with PBS and fetal calf serum-containing medium was added to all cultivated cell samples, and cells were incubated for another 48 h under the normoxic conditions. ELISA assays with rat cardiac myosin were performed to assess the expression of 2G42D7 ScFv.

The concentrations of ScFv antibody produced by the transfected H9C2 embryonic cardiocytes in different cell cultures are presented in Table 2. It follows from the data obtained that the transfection efficiency of hypoxic cells with IL was 3.5 times higher than for PL with hypoxic cells and 5.5 times higher than for PL with normoxic cells. (Better expression in PL-treated hypoxic cells than in PL-treated normoxic cells can be explained by increased plasmid delivery in hypoxic cells non-specifically sealed with PL).

Table 2
Expression of ScFv in different transfected cells (in μg/ml antibody concentration)

Cells and plasmid-containing liposomes	ScFv concentration by ELISA
Normoxic cells + PL	0.090 ± 0.001
Hypoxic cells + PL	0.212 ± 0.016
Hypoxic cells + IL	0.484 ± 0.023

Thus, we have solid reasons to believe that the use of drug-loaded cytoskeleton-specific IL in combination with artificially imposed hypoxic stress can facilitate intracellular delivery of drugs and genetic material. Further experiments in this direction are currently under way.

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