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# Monoclonal antibody to human endothelial cell surface internalization and liposome delivery in cell culture

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A monoclonal antibody (mAb), E25, is described that binds to the surface of cultured human endothelial cells. Upon binding E25 is rapidly internalized and digested intracellularly. Selective liposome targeting to the surface of the cells is performed using a biotinylated E25 antibody and an avidin-biotin system. Up to 30% of the cell-adherent liposomal lipid is internalized.

Monoclonal antibody; Avidin-biotin; Liposome; Targeting; Internalization; (Endiothelial cell)

## 1. INTRODUCTION

Liposomes can be used as containers to deliver physiologically active substances to living cells [1]. Despite the achievement of a high degree of liposome-cell association in some investigations [2,3], the problem of delivering the liposomal contents into the cell cytoplasm still exists. It is well known that in many cases only intracellular liposome delivery is required to cause effective pharmacological action of intraliposomal drugs on cells [4,5]. The problem is of particular importance for cells possessing low phagocytic activity, e.g. human vessel endothelial cells (EC). These cells are in direct contact with the blood and possess the unique property of exchanging macromolecular substances with underlying tissues [6]. Hence, the targeting of biologically active substances to EC may result in a considerable biological effect. The application of monoclonal antibodies against certain cell surface antigens can provide specificity and variety in liposome targeting and increased intracellular delivery [7].

Here, we describe mAb E25 which is rapidly internalized upon binding to cultured EC. A successful attempt was made to use this antibody as a mediator for cell surface binding and subsequent intracellular uptake of liposomes bound to mAb via an avidin-biotin bridge.

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*Abbreviations:* mAb, monoclonal antibody; EC, endothelial cell(s); HRPO, horseradish peroxidase; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; BAC-DPPE, biotinyl amidocaproyl-DPPE; HPA-DPPE, *p*-hydroxyphenylpropionylamido-DPPE; ILC, immunoliposome complex(es)

## 2. MATERIALS AND METHODS

### 2.1. *Antibodies and cells*

Murine mAb E25 (IgG1) were obtained by conventional techniques after immunization of mice with EC from human umbilical vein [8]. Purified mAbs E25 were labeled with $^{125}I$ [9] and modified with biotin-LC-hydroxysulfosuccinimide ester (Pierce). EC were cultivated as in [10].

### 2.2. *Internalization of mAbs*

A microtest plate with EC monolayers was ice-cooled, the wells rinsed with cold medium 199 (M199) supplemented with 10% calf serum (Flow Labs) and then 0.6 $\mu$g E25 in 30 $\mu$l M199 were added. After incubation for 1.5 h at 4°C, EC were washed with M199 and transferred to a $CO_2$ incubator at 37°C. After

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appropriate time intervals the incubation medium was removed and EC immediately cooled in ice. Then 0.15 μg HRPO-conjugated rabbit anti-mouse antibodies in 30 μl M199 were added. After 1 h at 4°C excess conjugate was washed out and the plate processed for determination of HRPO substrate absorbance ($A_{490}$) [11]. The rate of $^{125}$I-mAb degradation was determined following the appearance of radioactivity in the trichloroacetic acid-soluble fraction of the culture medium as described [12].

### 2.3. *Liposomes and ILC*

BAC-DPPE was synthesized according to [13]. $^{125}$I-labeled HPA-DPPE was prepared as in [14]. Liposomes composed of DPPC (Sigma), BAC-DPPE and HPA-DPPE at a molar ratio of 18:1:1 were sonicated in a probe-type sonicator (Lab-Line Instruments) for 5 min at 55°C and 50 W. The specific activity of the preparation was $3 \times 10^5$ cpm/μg total lipid. To prepare ILC 4 μg avidin D (Vector Labs) were added to 260 μl liposome suspension in Eagle's culture medium supplemented with 2 mg/ml bovine serum albumin (ECM, 40 μg lipid per ml) with subsequent addition of 3 μg biotinylated antibodies. ILC (34 μg lipid in 25 μl ECM) were added to ice-cold EC monolayers in a microtest plate and incubated for 1 h at 4°C. Non-bound ILC were removed by extensive washings. Fresh medium was added and EC incubated at 37°C for 1 h, followed by cooling in ice. 25 μl avidin-HRPO (Vector Labs) in ECM were added to each well and incubated for 1 h at 4°C. The EC monolayers were rinsed and processed for $A_{490}$ monitoring and counting of $^{125}$I.

## 3. RESULTS AND DISCUSSION

The mAb E25 binds to cultures of living and formaldehyde-treated EC from human umbilical vein. Binding of E25 to EC does not depend on monolayer preincubation (1 h, 25°C) with human plasma (fig.1) or incubation in the presence of whole blood (not shown). No binding of E25 to several other purified components of the extracellular matrix (human plasma fibronectin, type IV and V collagens) was observed.

Using an HRPO-labeled, second antibody it was revealed that surface-bound E25 rapidly disappears from the cell surface at 37°C (fig.2). The amount of surface-bound antibodies detectable was reduced by a factor of two within 30 min.

$^{125}$I-labeled mAbs E25 were incubated with EC and the kinetics of degradation of the internalized mAb was followed by analysis of the distribution of radioactive label in acid-soluble and acid-insoluble fractions of the culture medium. A continuous decrease in acid-precipitable radioactivity and concomitant accumulation of acid-soluble protein remnants were observed throughout the 180 min incubation period (fig.3). Cell-bound radioactivity, however, remained constant. Thus,

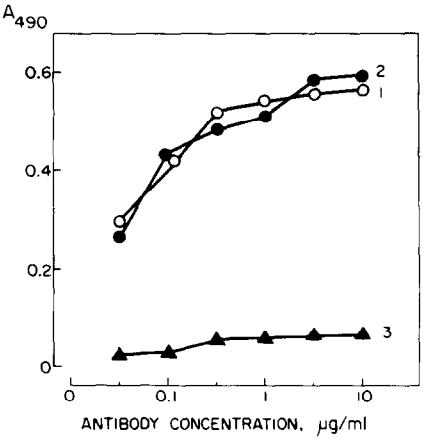



Fig.1. Binding of mAb E25 to cultured human EC (4°C) revealed using enzyme-labeled antimouse antibody. (1) Binding after 1 h incubation in human plasma, (2) binding without incubation with human plasma, (3) binding of non-specific mouse IgG to EC (control).

internalized antibodies were digested in intracellular compartments and degraded antibody fragments (acid-soluble fraction) could then be found in the culture medium. Moreover, the rate of antibody internalization was comparable to its rate of intracellular degradation. Similar tests with formaldehyde-fixed EC revealed no apparent changes in E25 binding to the cell surface, while all $^{125}$I radioactivity was precipitated by trichloroacetic acid. It seems reasonable to conclude that in

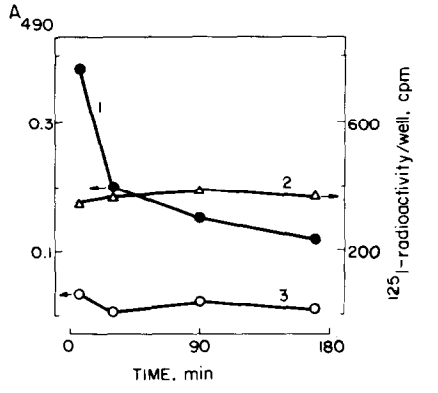



Fig.2. Internalization of $^{125}$I-mAb E25 by cultured human EC at 37°C as revealed with enzyme and radioactive labels. (1) EC surface-bound $^{125}$I-E25, (2) total EC-associated $^{125}$I-E25, (3) total binding of $^{125}$I-labeled mouse IgG (control).

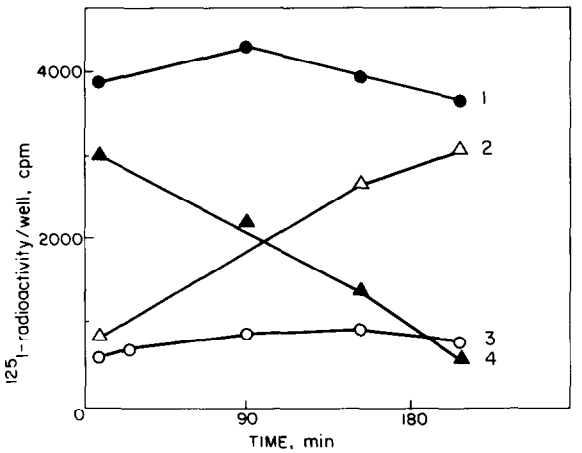



Fig.3. Kinetics of the degradation of $^{125}$I-E25 in cultured EC. (1) Total EC-associated $^{125}$I-E25, (2) $^{125}$I label in acid-soluble fraction, (3) $^{125}$I label in acid-precipitated fraction, (4) total $^{125}$I radioactivity in culture medium.

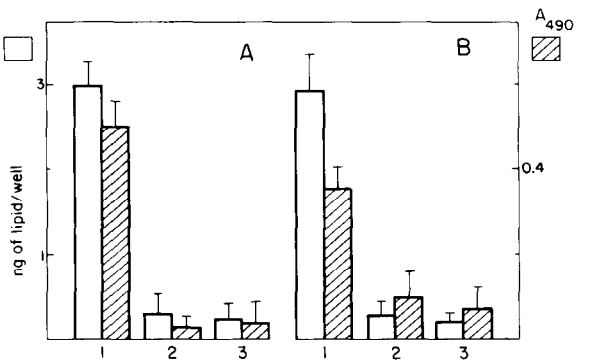



Fig.4. Total and surface binding of ILC to EC culture revealed by determinations of $^{125}$I and $A_{490}$ after incubation of ILC with EC at 4°C (A) and after 1 h incubation at 37°C (B).

living EC antibody-antigen complexes are subjected to active internalization and degradation.

Liposomes adhering non-specifically to the EC surface are not endocytosed. This was tested in experiments similar to those of the antibody internalization studies, except that avidin-HRPO was used instead of the second enzyme-labeled antibody. Hence, intracellular targeting of unmodified liposomes into EC cells is ineffective (fig.4).

The modification of liposomes with non-specific IgG in order to achieve targeting via Fc receptors as in [15] was also ineffective (fig.4). Liposomes were coated with biotinylated IgG via the avidin-biotin bridge technique. When biotinylated mAb E25 was used for ILC preparation (ILC-E25) it was demonstrated that the amount of EC-associated lipid increased 10-fold in comparison with liposomes bearing biotinylated, non-specific mouse IgG (ILC-IgG) or unmodified liposomes (fig.4). Up to 3 ng lipid per microtest well were bound as ILC-E25, equivalent to approx. 1000 liposomes of diameter 0.1 $\mu$m per cell [16]. When the temperature was increased from 4 to 37°C, a pronounced decrease in the amount of surface-bound ILC was observed. At 37°C about 30% of ILC-E25 are internalized, whereas only 70% of the ILC-E25 remain on the cell surface.

It should be mentioned that the targeted delivery described here is highly specific, since it is mediated by antigen-antibody interactions. This provides some advantages over the Fc receptor targeting systems reported earlier [15]. There are some indications in the literature that EC may be involved in targeted cancer chemotherapy aimed at preventing the neovascularization of tumors [17]. Since mAbs E25 bind to endothelium in the presence of serum or blood, the system described may be considered as a first step towards the targeting of biologically active substances into the EC in vivo. Detailed studies of the antigen recognized by mAb E25 and the structure of ILC are now in progress.

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