Immunotargeting of human cervical carcinoma xenograft expressing CA IX tumor-associated antigen by ^{125}I-labeled M75 monoclonal antibody*

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The aim of our present study was to explore a potential use of ^{125}I-labeled murine monoclonal antibody M75 that recognizes carbonic anhydrase IX (CA IX) in the immunotargeting of human cervical carcinoma xenografts in nude mice. CA IX is a hypoxia-inducible antigen, whose expression is significantly associated with carcinomas of the uterine cervix, whereas normal cervical tissue does not express CA IX protein. M75 monoclonal antibody was labeled with ^{125}I and used to quantify hypoxic induction of CA IX expression *in vitro* in HeLa human cervical carcinoma cells by immunoradiometric assay. HeLa cells showed inducible expression of CA IX *in vitro* by hypoxia (0.1% O_2) and various hypoxia mimicking agents (Co^{2+}, Ni^{2+}, Mn^{2+}, desferrioxamine, o-phenanthroline and $Na_2S_2O_4$). CA IX expression was also upregulated in the centre of HeLa multicellular clusters (spheroids) corresponding to the conditions of chronic hypoxia. For the immunotargeting study, ^{125}I-M75 was intravenously injected into immunodeficient mice bearing HeLa cervical carcinoma xenografts. Biodistribution profile showed selective and preferential accumulation of ^{125}I-M75 mAb in CA IX expressing HeLa xenografts in comparison with control unreactive ^{125}I-T111 antibody. Specificity was also confirmed by low uptake in CA IX negative C33A xenografts. In addition, CA IX expression in cervical carcinoma xenografts was analyzed by immunohistochemistry with M75. Detailed immunohistochemical analysis of HeLa xenograft sections revealed perinecrotically intensified expression of CA IX.

These results indicate that M75 mAb, recognizing CA IX antigen, has targeting properties which could be potentially useful in radioimmunodetection or radioimmunotherapy of human cervical carcinomas and derived metastases.

Key words: CA IX, cervical cancer, biodistribution, hypoxia, tumor targeting, M75 monoclonal antibody.

Cervical carcinoma is one of the most common cancer of women's reproductive tract. It represents a worldwide problem, causing 190,000 deaths yearly, with highest incidency in women living in developing countries [33].

Effective treatment of cervical cancer requires precise staging of a malignant lesion. Several examinations of the abdomen and pelvis, such as ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), lymphangiography, etc. are used for evaluation of anatomic extent of tumor infiltration. Examinations based on radioimmunodetection of cervical cancer may provide efficient choice for noninvaisve radiologic staging. Radiolabeled monoclonal antibodies (mAbs) selective for tumor-associated antigens have been effectively used in several clinical studies for radioimmunodetection of tumors by radioimmunoscintigraphy and for radioimmunotherapy with certain antitumor response [8]. Utilizations of mAbs in immunotargeting, resp. radioimmu-

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Abbreviations: BSA – bovine serum albumin; CA IX – human carbonic anhydrase IX protein; *CA9* – human carbonic anhydrase 9 gene; CIN – cervical intraepithelial neoplasia; CT – computed tomography; DFO – desferrioxamine; FIGO – staging nomenclature of the International Federation of Gynecology and Obstetrics; HIF1α – hypoxia-inducible factor 1α; %ID/g – uptake; mAb – monoclonal antibody; M75 – CA IX specific monoclonal antibody; LET – linear energy transfer; MRI – magnetic resonance imaging; oPE – o-phenanthroline; PBS – phosphate buffered saline; PET – positron emission tomography; pVHL – protein product of von Hippel-Lindau gene; RBE – relative biological effectiveness; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VEGF – vascular endothelial growth factor.

nodetection or radioimmunotherapy of cervical carcinomas were relatively rarely reported [6, 38, 39].

CA IX antigen is a transmembrane glycoprotein ectopically overexpressed in several types of cancer, while it is not expressed in normal counterparts. Normal cervical tissue without association with dysplasia or carcinoma are negative for CA IX, or only focally and weakly express CA IX, whereas cervical dysplastic or malignant tissues exhibit high association (higher than 90%) with CA IX expression [24]. CA IX was proposed as a valuable diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas [3, 24] and early diagnostic biomarker of cervical dysplasia [25]. In the extensive study of Liao and co-workers [24] M75 mAb was used for imunohistochemical analysis of CA IX expression in large collection of tissue samples and demonstrated presence of CA IX in 91% of CIN (82% of low-grade squamous intraepithelial lesions (condylomatous atypias and CIN-1: mild dysplasias) and 100% of high-grade ones (CIN-2: moderate dysplasias and CIN-3: severe dysplasias and carcinoma *in situ*)). Expression of CA IX in CIN positively correlates with grading, i. e. with probablity of malignant progression. In the case of cervical carcinomas, expression of CA IX was detected in 100% of squamous cell carcinomas, 93% of adenocarcinomas and in 100% of adenosquamous cell carcinomas [24]. Another studies showed a somewhat lower, but still significant expression of CA IX in adenosquamous carcinomas and adenocarcinomas [7] and in precursor lesions, CIN [34].

Significant association of CA IX expression with cervical cancer have led us to investigate a potential utility of M75 mAb in immunotargeting of cervical carcinoma. For this purpose, HeLa human cervical carcinoma xenografted nude mice were intravenously injected with ^{125}I-labeled M75 or control T111 mAbs and organ biodistribution analysis was performed.

Material and methods

Cell lines and culture conditions. HeLa cervical adenocarcinoma cells (ATCC CCL-2), C33A cervical carcinoma cells (ATCC HTB-31), SiHa squamous cervical carcinoma cells (ATCC HTB-35) and CaSki epidermoid cervical carcinoma cell line (ATCC CRL-7915) were grown in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. The HCE 16/3 immortalized cervical epithelial cells (provided by Prof. Vaheri, Helsinki) were grown in DMEM/F12 (1:1) medium with 2% (v/v) heat-inactivated fetal calf serum, 5 μg/ml insulin, 5 μg/ml EGF, 5 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 5 μg/ml transferin and 1.4 ng/ml 3,3´,5-triiodo-L-thyronine. All cell culture media were standardly supplemented with 2 mM L-glutamine and gentamicin (160 μg/ml). Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Hypoxic exposure. For experiments with hypoxic induction of CA IX, HeLa cells were plated at a low density $2.5x10^5$ cells per 6 cm dish, 1 day before hypoxic, or normoxic exposures. HeLa cells used for plating sparse density cell cultures, were maintained at least two passages at low density. The cells were then exposed to normoxia without or with 200 μM $CoCl_2$, 200 μM $MnCl_2$, 300 μM $NiCl_2$, 200 μM desferrioxamine (DFO), 150 μM o-phenanthroline (oPE) and 2 mM $Na_2S_2O_4$ (in sealed flasks) or to hypoxia for 16 h. Hypoxic conditions were achieved in a Napco 7000 incubator, which maintained an humidified atmosphere with 5% CO_2, 0.1% O_2 and the balance made up of nitrogen. Experimental incubations were done in normal growth medium for 16 h. Expression of CA IX was determined by immunoradiometric assay as described below.

Spheroid culture. HeLa cell culture (previously maintained at least for 96 h at low density) were seeded as a single cell suspension of $7.5x10^5$ cells per 10 cm petri dish coated previously with 0.7% agarose to obtain a non-adhesive surface. Culture medium was changed every 2 days. After 14 days of cultivation, spheroids were harvested, fixed in neutral-buffered formalin, and embedded in paraffin. Paraffin sections (4 μm thick) were dewaxed and used for immunohistochemical staining with M75 mAb or hematoxylin-eosin staining as described below.

Monoclonal antibodies, radiolabeling and immunoreactivity. The murine mAb M75 is an IgG2b that reacts with an epitope localised in a proteoglycan domain of CA IX [32, 40]. T111 mAb, an antibody specific for glycoprotein gB of HSV [5], was used as an isotype-matched, irrelevant control. Both mAbs were purified from hybridoma culture supernatant by affinity chromatography on protein A-Sepharose CL-4B column (Pharmacia Biotech). The purity of mAbs was verified by SDS-PAGE.

The monoclonal antibodies were labelled with $Na^{125}I$ by chloramine-T method as described by Hunter (1978) [17]. Briefly mAb (50 μg) was incubated with 18.5 MBq of $Na^{125}I$ (ICN Biomedicals, Inc.) and 20 μg of chloramine T in glass tubes for 1.5 min at 4 °C. Reaction was then stopped by addition of $Na_2S_2O_5$ and KI to a final concentration of 5 mM and 45 mM, respectively. All reagents were dissolved in 0.1 M phosphate buffer (pH 7.5) immediately before labeling. Free $Na^{125}I$ was removed by size exclusion chromatography using a Sephadex G50 column (Pharmacia Biotech). Specific activity was determined after precipitation by trichloroacetic acid. The immunoreactive fraction of ^{125}I-M75 was determined by the *in vitro* cell-binding assay, using HeLa cells corresponding to the biodistribution study. Immunoreactivity assay was based on the linear extrapolation of the radiolabeled antibody binding at an infinite excess amount of antigen according to the method of Lindmo [27]. Briefly, $2x10^5$ cpm of ^{125}I-M75 was incubated with increasing number of HeLa cells (trypsinized from dense cell culture) in a total volume of 1 ml of culture medium (in

triplicates) for 2 h at 37 °C. HeLa cell suspensions were then extensively washed and bound radioactivity was measured by Clinigamma counter (Pharmacia LKB) at 60 s counting intervals. Nonspecific binding was determined in the presence of 100-fold molar excess of unlabeled M75 and substracted from the total binding to calculate a specific binding. The saturation binding data were transformed and plotted as the reciprocal of the cell number against the reciprocal of the fraction bound. Obtained data were fitted with a least squares linear regression method. The immunoreactive fraction was calculated as the reciprocal value of the fraction bound intercept.

Immunoradiometric assay. The cells were incubated with ^{125}I-M75 mAb $5x10^5$ cpm.ml^{-1} at 37 °C for 2 h and then washed. The cultures were then extracted by RIPA lysis buffer (1% Triton X-100, 0.1% sodium desoxycholate, 1 mM PMSF, 20 μg/ml trypsin inhibitor, 1 mM ethylenediaminetetraacetic acid in PBS) for 30 min at room temperature, extracts were centrifuged and concentration of proteins in supernatants was determined by bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's instructions. Radioactivity in cell extracts was measured by Clinigamma counter (Pharmacia LKB) at 60 s counting intervals. Radioactivities of samples were normalized to protein amount and results were expressed as means with corresponding standard deviations.

Western blotting. The confluent cell monolayers were extracted by RIPA lysis buffer for 30 min at room temperature. The extracts were then centrifuged (15 min at 13 000 rpm) and concentration of proteins was determined by BCA assay (Pierce) according to the manufacturer's instructions. Samples were separated by electrophoresis on a 10% SDS-PAGE gel, blotted onto PVDF membrane (Immobilon^{TM}-P, Millipore, Bedford). The membrane was incubated with ^{125}I-labeled M75 mAb and then exposed to X-ray film (FUJI photo film, Japan).

Histological and imunohistochemical analysis of tumor sections. Dissected tumors were fixed in neutral-buffered formalin and embedded in paraffin according to standard histological procedures. Dewaxed and rehydrated sections (4 μm) were used for histological and immunohistochemical analysis. For immunohistochemistry, slides were blocked by incubation in 50 mM Glycine in PBS two times for 10 min at room temperature and then in 1% BSA in PBS for 30 min at 37 °C. Endogenous peroxidase was blocked by 5 min incubation with 3% H_2O_2 in distilled water at room temperature. Slides were then washed with PBS and incubated with biotinylated M75 (0.7 μg.ml^{-1} in 0.5% BSA) for 1 h at 37 °C. Sections were then extensively washed with 0.2 % Tween-20 in PBS and incubated with streptavidin-HRP (2 μg.ml^{-1} in 0.5% BSA) for 1 h at 37 °C. Diaminobenzidine was used as a chromogenic substrate and sections were counterstained with Mayer's hematoxylin.

Biodistribution study. CD-1 nu/nu mice, 6–8 weeks of age (Charles River, Germany) were injected subcutaneously with $5x10^5$ of HeLa cells or $1.5x10^7$ of C33A cells. When the tumors had achieved approximately 0.5 cm diameter (after ~2–3 weeks), 0.37 MBq of ^{125}I labeled M75, or control T111 monoclonal antibody was administered intravenously via the tail vein. Groups of four mice that received the ^{125}I-M75 or ^{125}I-T111 were sacrificed by cervical dislocation and dissected 48 h after injection. The ^{125}I radioactivity of tumor and tissues of interest were measured by Clinigamma counter (Pharmacia LKB) at 60 s counting intervals. Uptakes were corrected for radiodecay and expressed as percentage of injected dose per gram of tissue (%ID/g).

Results

CA IX expression in cervical carcinoma cell lines. Expression of CA IX in cervical cells and in cell lines derived from cervical carcinomas was analyzed by Western blotting (Fig. 1). HCE16/3 immortalized ektocervical cells displayed weak expression of CA IX. SiHa, HeLa and CaSki cervical

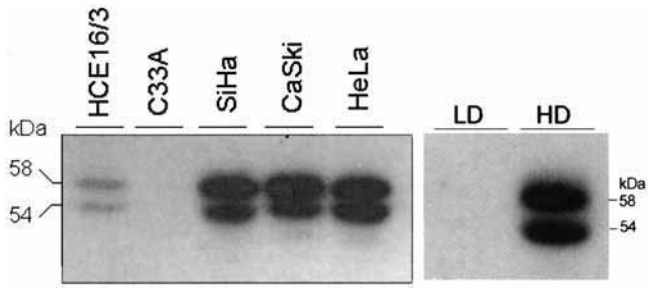

Figure 1. CA IX expression in cell lines derived from the cervix uteri analyzed by Western blotting. A, expression of CA IX in confluent cell cultures of HCE16/3, C33A, SiHa, CaSki, and HeLa. B, expression of CA IX in sparse (LD) and dense (HD) HeLa cells. 40 μg of protein extracts from dense cell cultures were loaded per lane.

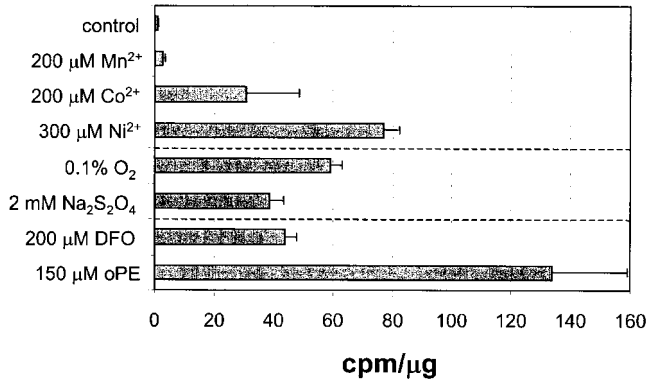

Figure 2. Induction of CA IX expression in HeLa cells by hypoxia, hypoxia mimicking transition metals and iron chelators. Low density HeLa cells were exposed to normoxia (21% O_2) with indicated agents or to hypoxia (0.1% O_2) for 16 h and than CA IX expression was analyzed by immunoradiometric assay, n=3.

Figure 3. CA IX expression in multicellular HeLa human cervical carcinoma spheroid. HeLa spheroids were fixed in neutral-buffered formalin and paraffin embedded. A, the expression of CA IX in spheroid sections was immunohistochemicaly detected with M75 mAb and counterstained with Mayer's hematoxylin. As can be seen CA IX is expressed predominantly in the centre of the spheroid. B, HeLa spheroid microphotograph. C, CA IX expression in HeLa spheroids analyzed by Western blotting.

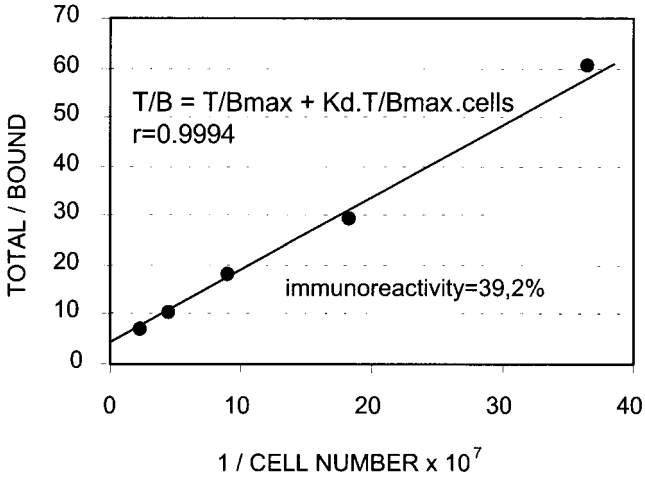

Figure 4. Determination of immunoreactive fraction of ^{125}I-M75 by extrapolation to antibody binding at infinite antigen excess. 2x10^5 cpm of ^{125}I-M75 mAb (with or without 100-fold molar excess of unlabeled M75) was incubated with increasing number of HeLa cells at 37 °C for 2 h. The cells were then washed and counted in a gamma counter. Reciprocal values of obtained data were fitted with a least squares linear regression method and immunoreactivity was calculated as the reciprocal value of the fraction bound intercept.

carcinoma cell lines showed strong expression of CA IX, whereas C33A cell line does not express CA IX due to deletion of locus encoding CA IX [26]. HeLa cell line was selected for further *in vitro* characterization of hypoxic induction of CA IX expression and for *in vivo* study of M75 mAb mediated targeting of cervical carcinoma xenografts in nude mice. And C33A cell line was used as CA IX negative control for ^{125}I-M75 biodistribution study.

Induction of CA IX expression in vitro by hypoxia, hypoxia mimicking agents and iron chelators. Hypoxia inducible expression of CA IX was demonstrated in the low density plated HeLa cells, which do not express CA IX (Fig. 1B). Expression of CA IX in dense HeLa cell culture (Fig. 1A and 1B) is caused by a high cell density-mediated lowered oxygen tension [20]. As can be seen (Fig. 2) expression of CA IX was induced with hypoxic exposure (0.1% O_2), or by stimulation with hypoxia mimicking cations of transition metals Co^{2+}, Ni^{2+} and slightly by Mn^{2+}. Hypoxic induction was also achieved by incubation with $Na_2S_2O_4$ (2 mM) which is able to effectively eliminate oxygen from the culture medium. Induction of CA IX by Co^{2+} and Ni^{2+} divalent cations corresponds with possible displacement of oxygen from a putative heme-containing sensor [12]. Recent observation also suggests that Co^{2+} and Ni^{2+} ions bind to cullin-2 and affects the function of E3-ligase complex [21] and by this mechanism stabilise HIF1α and may induce CA IX expression. Strong upregulation of CA IX expression was achieved by iron-chelators o-phenanthroline (structural analog of 2,2'-dipyridyl, 150 μM) and desferoxamine (200 μM). Iron chelators mimic hypoxia by interference with an oxygen-dependent proline and asparagine hydroxylation of HIF1α [23] or probably by exhaustion of Fe(II) from Fenton reaction [10].

Multicellular cervical carcinoma HeLa cell spheroids were studied as a model of micrometastases with chronic hypoxia. Many studies well document gradient profiles of oxygen tension across the spheroids [1]. Therefore, interior of large multicellular cluster (spheroid) simulates hypoxic areas of tumor located distantly from blood capillary or near to defective blood capillary. CA IX expression was observed especially in those spheroids whose diameter was greater than approximately 200 μm. CA IX expression was predominantly localized in the central rim of HeLa spheroids and only limited expression was present in the peripheral cell layers (Fig. 3A), likewise as it was observed in the case of another hypoxia induced protein, VEGF [13].

Radiolabeling efficiency and immunoreactivity. M75 and T111 mAbs were radioiodinated with chloramine T method to specific activities of 0.205 MBq.μg^{-1} and 0.158 MBq.μg^{-1}, respectively. Radiolabeling usually achieved ^{125}I-isotope

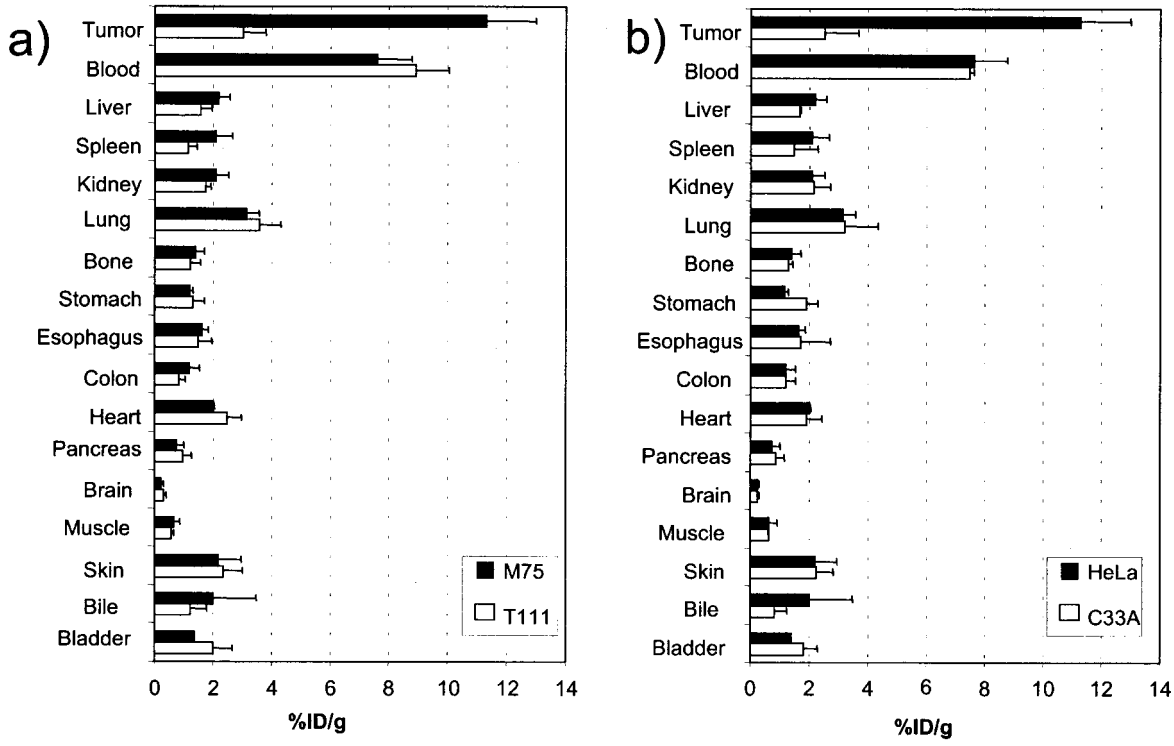

Figure 5. Biodistribution profiles of ^{125}I-labeled M75 and T111 mAbs. A, comparision of uptakes of ^{125}I-M75 mAb (black bars) versus ^{125}I-T111 mAb (open bars) in HeLa tumor xenograft and normal tissues. B, comparison of uptakes of ^{125}I-M75 mAb in HeLa xenografted (black bars) versus C33A xenografted (open bars) in tumor and normal tissues. Groups of mice (n=4) were intravenously injected with 0.37 MBq dose of appropriate mAb and 48 h later dissected and analyzed.

incorporation of 62% for M75 and of 43% for T111, and amounts of free ^{125}I in the radiolabeled mAb preparations after size exclusion chromatography on Sephadex G50 was less than 0.2%. The immunoreactive fraction of ^{125}I-M75 mAb after radioiodination determined by linear extrapolation of antibody binding to an infinite amount of antigen (Fig. 4) was 39.2%. The immunoreactivity of ^{125}I-T111 was not determined (irrelevant).

Biodistribution study of ^{125}I-M75 in human cervical carcinoma xenografted nude mice. The biodistribution data of ^{125}I-labeled M75 mAb in athymic nude mice bearing HeLa cervical carcinoma xenograft are presented in Figure 5. The uptake (%ID/g) of ^{125}I-M75 in HeLa tumor xenograft was distinct in comparison with normal tissues 48 h after injection (Fig. 5a). ^{125}I-M75 was significantly accumulated by HeLa xenograft, whereas normal tissues (liver, spleen, kidney, lung, bone, stomach, esophagus, colon, heart, pancreas, brain, muscle, skin, bile and bladder) had lower accumulation of the radioactivity.

When ^{125}I-T111 was administred as a control antibody, biodistribution pattern in normal organs was similar to M75, with the exception of the HeLa xenograft (Fig. 5a). Injection of ^{125}I-M75 into C33A xenografted nude mice further confirmed specificity of M75 localization (Fig. 5b). Uptake of ^{125}I-M75 in CA IX negative C33A xenografts was significantly lower when compared with CA IX positive HeLa tumors. And therefore, accumulation of ^{125}I-M75 in HeLa xenografts was not caused by passive localization, but as a result of specific antibody-antigen interaction.

Histological and immunohistochemical analysis of tumor sections. H-E staining of both HeLa and C33A tumor xenograft sections showed extensive content of eosinophilic regions (Fig. 6a and 6b). These areas were deeply infiltrated predominantly by mononuclear leukocytes and partialy by polymorphonuclear leukocytes and macrophages. Tumor cells in these regions displayed cellular degeneration, such as pycnotic nucleus, karyolysis and karyorrhexis – all features of necrosis. The average extent of necrosis in HeLa xenografts was approximately 30%. Detailed immunohistochemical analysis revealed perinecrotically intensified expression of CA IX in HeLa xenograft sections (Fig. 6d), whereas control sections of C33A xenograft were immunohistochemicaly negative for CA IX expression (Fig. 6c). CA IX staining pattern in HeLa tumor xenograft sections reflects tumor cell heterogeneity, xenograft composition – presence of CA IX negative stroma and corresponds with hypoxia-inducible expression of CA IX.

Figure 6. Hematoxylin-eosin staining of C33A (a) and HeLa xenograft sections (b). Imunohistochemical analysis of CA IX expression in neutral-buffered formalin-fixed paraffin embedded C33A (c) and HeLa (d) xenograft sections using the avidin-biotin technique with 3,3'-diaminobenzidine as chromogen. Note: n-necrosis. Tumor sections were stained with biotinylated M75 mAb followed by streptavidine-HRP and than counterstained with Mayer's hematoxylin.

Discussion

Despite the existence of effective screening procedures based on Papanicolaou exfoliative cytologic test, cervical cancer still remains a significant cause of mortality [33]. Early stages of cervical cancer (FIGO Ib, IIa) are usually surgicaly treated with radical hysterectomy and pelvic/periaortic lymphadenectomy. The more advanced cases (FIGO IIb and greater) require primary treatment with radiotherapy (external-beam radiation and intracavitary brachytherapy). The late stages of cervical carcinoma have considerably unfavorable survival rates. Cervical carcinomas are radioresponsive tumors with curability adversely affected by hypoxia [35]. In generaly, hypoxic tumors respond poorly to chemotherapy and radiotherapy [4]. It is known for a long time that tumor oxygenation is essential for efficient radiotherapy of tumors with sparsely ionizing radiation (x- or γ-rays) with low linear energy transfer (LET), because oxygen plays crucial role in fixation of radiation lesions [36]. In addition, intratumoral hypoxia has significant influence on malignant progression of solid tumors [15] and thus it is associated with poor local control and survival of patients after therapy [4]. Hypoxia as a common feature of the most human cervical carcinomas [15] has been reported to be a predictive factor for treatment failure and survival in cervical cancer [14, 22]. Patients with more hypoxic cervical carcinomas have significantly increased risk of nodal and distant metastases, and have greater frequency of subsequent relapse than those with better oxygenated tumors [11, 15]. Hypoxic cervical cancer associated with low-apoptotic index demonstrate aggressive behavior with extremely poor outcome for clinical patients [16]. The relevance of tumor hypoxia in prognosis of cancer treatment lead to development of hypoxia imaging techniques [9] and bioreductive drugs [19]. Immunotargeting of hy-

poxic areas of solid tumors mediated by specific monoclonal antibody represents a novel alternative approach for detection of tumors, and of intratumoral hypoxia, respectively. In addition, targeted delivery of therapeutic agents conjugated to this monoclonal antibody to the hypoxic regions of tumors may enhance the efficiency of anticancer therapy.

CA IX is a transmembrane carbonic anhydrase whose expression is closely associated with carcinomas of cervix, esophagus, kidney, colorectum, lung, breast, bladder and skin [24, 37]. In normal tissues, CA IX expression was observed only in epithelia of gastrointestinal tract [24, 31]. Therefore CA IX appears as a suitable target antigen for radioimmunodetection and radioimmunotherapy of multiple epithelial malignancies. In the recent time, expression of *CA9* has been found strongly upregulated by low tension of oxygen [2, 18, 37]. Minimal promoter of *CA9* gene contains aHIF-1 dependent hypoxia response element (HRE) and transcription of *CA9* is tightly regulated by oxygen controled HIF-1/pVHL mechanism [37]. Although, real function of CA IX is not known, carboanhydrase activity of CA IX probably help to maintain intracellular acidobasic homeostasis in the conditions of hypoxic tumor microenvironment. CA IX was proposed as an intrinsic marker of intratumoral hypoxia [2] also for hypoxic cells of cervical carcinoma [28, 30]. CA IX expression and hypoxia marker pimonidazole showed excellent colocalization in sections from tumors of patients with cervical carcinomas [30]. Also direct measurements of tumor oxygenation with polarographic needle electrode in patients with cervical carcinoma showed significant positive correlation between CA IX expression and the level of tumor hypoxia [28]. This clinical study confirmed that CA IX as a intrinsic marker of intratumoral hypoxia is a prognostic indicator of overall survival after radiation therapy [28]. Positive relationship between CA IX expression and radioresistance was also observed in murine model with SiHa cervical carcinoma xenografts [30]. Altogether, these reported data suggest that CA IX expression reflects intratumoral hypoxia in cervical carcinomas and correlates with radioresistance of tumor cells.

In the present study we investigated targeting potential of ^{125}I-M75 mAb in nude mice bearing cervical tumor xenografts. Nude mice were xenografted with HeLa cell line which exhibited strong inducibility of CA IX expression by hypoxia and hypoxia mimicking agents *in vitro*. Analysis of biodistribution profile of ^{125}I-M75 in normal and tumor tissues showed selective accumulation in CA IX positive HeLa xenografts. Despite relatively low immunoreactivity of ^{125}I-M75 obtained after radiolabeling with chloramine T, significant tumor uptake was achieved and may be probably further improved by utilization of another radioiodination methods with milder oxidation conditions, such as iodogen, or by indirect radiolabeling with Bolton and Hunter's acylating agent. Elevated plasmatic activity of ^{125}I-M75 may be reduced by elimination of unbound M75 from circulation by extracorporeal immunoadsorption or by pretargeting strategy. The tumor specific uptake of ^{125}I-M75 reflects CA IX expression, respectively extension of hypoxic areas and therefore, radioimmunodetection of CA IX may provide significant diagnostic and prognostic information. High level of CA IX expression (induced by large intratumoral hypoxia) detected by radiolocalization of M75 mAb may have indicative significance for selection of patients who need more radical approach then radiotherapy alone or who can undergo hyperoxic treatment [29] or therapy with bioreductive drugs [19].

Tumor selective accumulation of M75 also suggests possible utilization of this antibody in targeted delivery of therapeutic agents (radioisotopes, cytotoxic drugs, conditionaly toxic enzymes etc.) to the tumor cells. M75 mediated targeted delivery of radioisotopes can selectively improve radiation dose to hypoxic regions of tumors. M75 labeled with α-particle emitters (^{212}Bi, ^{213}Bi and ^{211}At) could be potentially used for radioimmunotherapy of cervical carcinomas, because immunotargeting of α-emission with high LET (~100 keV.μm^{-1}) (whose relative biological effectiveness (RBE) is independent on oxygenation) to CA IX positive hypoxic areas can solve problem of radioresistance of hypoxic tumor cells to x-, γ- and β-radiation with low LET (0.1–3 keV.μm^{-1}).

M75 or other CA IX specific monoclonal antibodies effective in radioimmunotargeting of cervical malignancies and metastases could be potentially usefull also for tumor imaging and radioimmunoguided surgery approach. Pattern of spread of cervical carcinoma involves direct invasion to the adjacent vaginal fornices or to the paracervical and parametrial tissues and dissemination occurs also through the lymphatic network or by hematogenous route in advanced stages. The most frequent metastatic organs are liver, lungs, brain, bones, mediastinal and supraclavicular lymph nodes. Thus radioimmunoscintigraphy of the pelvis or whole body with M75 may help to define the extent of tumor growth and guide therapy decisions. Other imaging modalities, MRI and PET with appropriately labeled M75 may further increase the accuracy of detection and noninvasive staging of cervical carcinomas.

In conclusion, this preliminary study indicates that M75 mAb is a potential tool for radioimmunotargeting of cervical cancer and targeting properties of this antibody will be studied further.

References

[1] Acker H, Carlsson J, Holtermann G, Nederman T, Nylen T. Influence of glucose and buffer capacity in the culture medium on growth and pH in spheroids of human thyroid carcinoma and human glioma origin. Cancer Res 1987; 47: 3504–3508.

[2] Beasley NJ, Wykoff CC, Watson PH, Leek R, Turley H, Gatter K, Pastorek J, Cox GJ, Ratcliffe P, Harris AL. Carbonic anhydrase IX, an endogenous hypoxia marker: expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. Cancer Res 2001; 61: 5262–5267.

[3] Brewer CA, Liao SY, Wilczynski SP, Pastoreková S, Pastorek J, Zavada J, Kurosaki T, Manetta A, Berman ML, DiSaia PJ, Stanbridge EJ. A study of biomarkers in cervical carcinoma and clinical correlation of the novel biomarker MN. Gynecologic Oncology 1996; 63: 337–344.

[4] Brizel DM, Sibley GS, Prosnitz LR, Scher RL, Dewhrist MW. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 1997; 38: 285–289.

[5] Bystrická M, Vančíková M, Kasalová M, Russ G, Rajčáni J, Košťál M, Poláková K. Type – common and type – specific monoclonal antibodies to Herpes simplex virus type 1 and 2. Acta Virol 1991; 35: 152–164.

[6] Chen FM, Taylor CR, Epstein AL. Tumor necrosis treatment of ME-180 human cervical carcinoma model with 131I-labeled TNT-1 monoclonal antibody. Cancer Res 1989; 49: 4578–4585.

[7] Costa M J, Ndoye A, Trelford JD. MN protein immunolocalization in uterine cervix carcinoma with glandular differentiation. A clinicopathologic study of a new cancer-specific biomarker. Int J Surg Pathol 1995; 3: 73–82.

[8] DeNardo SJ, Kroger LA, DeNardo GL. A new era for radiolabeled antibodies in cancer? Curr Opin Imunol 1999; 11: 563–569.

[9] Dolbier RW, Li AR, Koch CJ, Shiue CY, Kachur AV. [^{18}F]-EF5, a marker for PET detection of hypoxia: synthesis of precursor and new fluorination procedure. Applied Radiation and Isotopes 2001; 54: 73–80.

[10] Ehleben W, Porwol T, Fandrey J, Kummer W, Acker H. Cobalt and desferrioxamine reveal crucial members of the oxygen sensing pathway in HepG2 cells. Kidney Int 1997; 51: 483–491.

[11] Fyles A, Milosevic M, Hedley D, Pintilie M, Levin W, Manchul L, Hil RP. Tumor hypoxia has independent predictor impact only in patients with node-negative cervix cancer. J Clin Oncol 2002; 20: 610–615.

[12] Goldberg MA, Dunning SP, Bunn HF. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science 1998; 242: 1412–1415.

[13] Hemmerlein B, Kugler A, Ozisik R, Ringert R-H, Radzun H-J, Thelen P. Vascular endothelial growth factor expression, angiogenesis, and necrosis in renal cell carcinomas. Virchows Arch 2001; 439: 645–652.

[14] Höckel M, Knoop C, Schlenger K, Vorndran B, Baussmann E, Mitze M, Knapstein PG, Vaupel P. Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. Radiother Oncol 1993; 26: 45–50.

[15] Höckel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996; 56: 4509–4515.

[16] Höckel M, Schlenger K, Höckel S, Vaupel P. Hypoxic cervical cancers with low apoptotic index are highly agressive. Cancer Res 1999; 59: 4525–4528.

[17] Hunter WM. Radioimmunoassay. In: Weir DM, editor. Handbook of Experimental Immunology. Blackwell scientific publications, Oxford/London/Edinburgh/Melbourne, 1978: 14.1–14.40.

[18] Ivanov SL, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 2001; 158: 905–919.

[19] Jaffar M, Wiliams KJ, Stratford IJ. Bioreductive and gene therapy approaches to hypoxic diseases. Advanced Drug Delivery Reviews 2001; 53: 217–228.

[20] Kaluz S, Kaluzová M, Chrastina A, Olive PL, Pastoreková S, Pastorek J, Lerman MI, Stanbridge EJ. Lowered oxygen tension induces expression of the hypoxia marker MN/Carbonic anhydrase IX in the absence of hypoxia-inducible factor 1α stabilization. Cancer Res 2002; 62: 4469–4477.

[21] Kanaya K, Tsai AL, Kamitani T. Cobalt- and nikel-binding property of cullin-2. Biochem and Biophys Res Com 2002; 290: 294–299.

[22] Knocke T, Weitmann H, Feldman H, Selzer E, Potter R. Intratumoral pO_2 measurements as predictive assay in the treatment of carcinoma of the uterine cervix. Radiother Oncol 1999; 53: 99–104.

[23] Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. Science 2002; 295: 858–861.

[24] Liao SY, Brewer C, Závada J, Pastorek J, Pastoreková S, Manetta A, Bermann ML, DiSaia PJ, Stanbridge EJ. Identification of MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. Am J Pathol 1994; 145: 598–609.

[25] Liao SY, Stanbridge EJ. Expression of MN antigen in cervical Papanicolaou smears is an early diagnostic biomarker of cervical dysplasia. Cancer Epidemiology, Biomarkers and Prevention 1996; 5: 549–557.

[26] Lieskovská J, Opavský R, Žáčiková Ľ, Glasová M, Pastorek J, Pastoreková S. Study of *in vitro* conditions modulating expression of MN/CA IX protein in human cell lines derived from cervical carcinoma. Neoplasma 1999, 46: 17–23.

[27] Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J Immunol Methods 1984; 72: 77–98.

[28] Loncaster JA, Harris AL, Davidson SE, Logue JP, Hunter RD, Wycoff CC, Pastorek J, Ratcliffe PJ, Strarford IJ, West CML. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res 2001, 61: 6394–6399.

[29] Nordsmark M. Direct measurements of tumor-tissue pO_2. A way of selecting patients for hyperoxic treatment. Strahlenther Onkol 1996; 172 Suppl 2: 8–9.

[30] Olive PL, Aquino-Parsons C, MacPhail SH, Liao SY, Raleigh JA, Lerman MI, Stanbridge EJ. Carbonic anhydrase

9 as an endogenous marker for hypoxic cells in cervical cancer. Cancer Res 2001; 61: 8924–8929.

[31] Pastoreková S, Parkkila S, Parkkila AK, Opavský R, Zelník V, Saarnio J, Pastorek J. Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementery DNA sequence and expression in human and rat alimentary tracts. Gastroenterology 1997; 112: 398–408.

[32] Pastoreková S, Závadová Z, Košťál M, Babušíková O, Závada J. A novel quasi-viral agent, MaTu, is a two-component system. Virology 1992; 187: 620–626.

[33] Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 1999; 83: 18–29.

[34] Resnick M, Lester S, Tate JE, Sheets EE, Sparks C, Crum CP. Viral and histopathologic correlates of MN and MIB-1 expression in cervical intraepithelial neoplasia. Hum Pathol 1996; 27: 234–239.

[35] Rofstad EK, Sundfor K, Lyng H, Tropé CG. Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis. Br J Cancer 2000; 83: 354–359.

[36] Wright EA, Howard-Flanders P. The influence of oxygen on the radiosensitivity of mamalian tissues. Acta Radiol 1957; 48: 26–32.

[37] Wykoff CC, Beasley NJP, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. Hypoxia inducible regulation of tumor associated carbonic anhydrases. Cancer Res 2000; 60: 7075–7083.

[38] Yeh MY, Roffler SR, Yu MH. Doxorubicin: monoclonal antibody conjugate for therapy of human cervical carcinoma. Int J Cancer 1992; 51: 274–282.

[39] Yuan CC, Tsai lC, Lee TW, Ng HP, Yeh SH. Radioimmunodetection of human cervical carcinoma xenograft by 111In-labeled monoclonal antibody Mab Cx-99. Int J Gynaecol Obstet 1995; 49 Suppl: S33–S38.

[40] Závada J, Závadová Z, Pastorek J, Biesová Z, Ježek K, Velek J. Human tumour-associated cell adhesion protein MN/CA IX: identification of M75 epitope and of the region mediating cell adhesion. Br J Cancer 2000; 82: 1808–1813.