Direct Validation of Aptamers as Powerful Tools to Image Solid Tumor

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Visualization of cancer cells requires distinguishing malignant from normal cells by objective criteria with high specificity. For several years, tumor markers expressed on the surface of cancer cells have been characterized as cancer signatures, and their labeling with specific imaging probes has revolutionized cancer diagnosis. This specific labeling is also an important tool in surgery tumor ablation. The present study considers the tumor labeling potential of an aptamer that specifically recognizes the epithelial cancer biomarker mucin1 (MUC1). This anti-MUC1 aptamer was investigated *in vitro* in a three-dimensional (3D) environment and compared to an anti-MUC1 antibody for its capacity to visualize cancer cells. Multicellular spheroids of breast cancer MCF-7 cells were used as tumor models and anti-MUC1 fluorescent aptamer and antibody were visualized by fluorescence imaging. Results showed that the antibodies interacted only with cells located on the surface of the spheroid, whereas the anti-MUC1 aptamers were able to penetrate inside these 3D tumor models and thereafter internalized into the cancer cells. Due to their lack of immunogenicity and their facility to be chemically modified, aptamers may replace advantageously the use of antibodies in diagnosis based on imaging setup thanks to their specific detection of cancer cells without invasive surgical procedures or during clinical intraoperative intervention.

Introduction

AMONG THE CANCER BIOMARKERS that have already been identified, most of them are surface antigens over-expressed in cancer cells. These surface biomarkers are ideal targets for imaging because of their direct accessibility. The use of imaging probes directed against specific biomarkers expressed on the surface of cancer cells has allowed specific visualization of tumor cells that has helped guide surgeons during clinical intraoperative intervention. The prostate-specific membrane antigen, the carcinoembryonic antigen (CEA) and some mucin-type glycosated proteins such as mucin1 (MUC1) in epithelial breast cancers or MUC16 in epithelial ovarian cancers have already been identified for targeting tumor cells (Cibiel et al., 2012). MUC1 is currently one of the major cancer biomarkers. It is highly expressed by the majority of cancers cells and, in particular, by primary and metastatic breast cancers (Da Pieve et al., 2009). Recently, a targeted active immunotherapy protocol was designed by the Transgene Company to treat MUC1 expressing solid tumor using a recombinant vaccinia virus and some antibodies against MUC1 have been developed to visualize cancer cells or to target metastatic cancers (Horm and Schroeder, 2013).

For many years, antibodies were considered as the most specific probes for tumor imaging due to their high affinity and their good specificity for surface receptors (Gao et al., 2004; Cohen and Margel, 2012; Kaur et al., 2012). Through extensive studies, their usefulness during intraoperative surgery has been demonstrated (Kaushal et al., 2008; McElroy et al., 2008; Terwisscha van Scheltinga et al., 2011).

Despite their specificity and affinity towards their targets, antibodies present two major restrictions for *in vivo* tumor imaging: (1) their immunogenicity and (2) the difficulty to obtain the chemical modifications needed for their fluorescent labeling. During the last 20 years, antibody fragments (scFv), peptides and aptamers have been developed as alternative approaches to overcome these limitations. But for scFv and peptides, their stability remains a major problem for their use in *in vivo* studies (Worn and Pluckthun 2001; Jin et al., 2013).

Nucleic acid aptamers are nowadays considered as nucleic-antibody analogues and have emerged as new targeting probes with excellent potential for theranostic applications (Cerchia and de Franciscis, 2010; Ruigrok et al., 2011; Zhu et al., 2012). They are classically generated by the SELEX method from synthetic single-stranded DNA or RNA

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libraries and are selected for their ability to bind their target antigens with strong affinity and specificity (Mayer, 2009). Because of their small size (< 30 kDa) and their properties as nucleic acids, aptamer synthesis is very well controlled, and a lot of chemical modifications can be introduced in order to get better stabilization, to reduce renal clearance or to conjugate different agents such as imaging dyes. Moreover, their production is at low cost and reproducible, decreasing batch-to-batch variability. An increasing number of labeled aptamers, conjugated to fluorescent molecules, have been developed in order to validate their use in tumor-specific imaging for diagnosis and potentially for intraoperative surgery (Charlton et al., 1997; Gao et al., 2004; Shi et al., 2011; Cibiel et al., 2012; Kim et al., 2012; Bellard et al., 2013).

The anti-MUC1 aptamer is very well described and shows a high affinity (0.135 nM) and good specificity for its target (Ferreira et al., 2006; Baouendi et al., 2012). This aptamer has been shown to successfully recognize and bind to native MUC1 at the surface of the MCF-7 breast cancer cell line in cell culture (Yu et al., 2011; Hu et al., 2012; Wu et al., 2012; Cai et al., 2013) and in tumor-bearing mouse (Da Pieve et al., 2009; Orava et al., 2010; Kurosaki et al., 2012). However, no direct comparison between MUC1 aptamer and anti-MUC1 antibody has been reported.

To better understand the potential of fluorescent aptamers versus fluorescent antibodies, the present study evaluates the use of a fluorophore-labeled anti-MUC1 aptamer as an imaging agent using spheroid assemblies as model tumors. Spheroids as three-dimensional (3D) *in vitro* models provide a relevant alternative to both complex *in vivo* whole organism approaches and two-dimensional (2D) culture with its limitations. Obviously, tissue-specific architecture and organization as well as mechanical and biochemical cues are absent from classical monolayer culturing techniques. Developing 3D cell culture models not only reduces the gap between *in vitro* cell culture and *in vivo* tissues, but also complies with the ethical principles of animal research (Gibot and Rols, 2013). Therefore, 3D models appear appropriate for evaluating the labeling and penetration efficiency of aptamers.

We showed, using a 3D model spheroid of MCF-7 cells, that MUC1 aptamer is a good alternative probe to anti-MUC1 antibody in imaging studies. Coupled with fluorophores, both aptamers and antibodies were able to label cancer cells in the spheroid. Whereas antibodies interact with MUC1 target only at the surface of the spheroid, aptamers were able to penetrate deeply in the core of these 3D tumor models.

We clearly demonstrated the accessibility of inner cells in the spheroid to MUC1 aptamers and an efficient internalization in all cells of the spheroids.

Finally, we propose that aptamers may advantageously replace antibodies in diagnostic imaging, where they could specifically detect cancer cells without invasive surgical procedures, or in clinical intraoperative intervention, where they would guide surgeons in removing tumors.

Materials and Methods

Aptamer sequences, products, and reagents

The Cy3-5'-labeled-mucin1 DNA aptamer (Cy3-MUC1 aptamer) was purchased from Sigma-Aldrich [5'-GGGAG ACAAGAATAAACGCTCAAGCAGTTGATCCTTTGGA TACCCTGGTTCGACAGGAGGCTCACAACAGGC-3']

(dissociation constant, K_d : 0.135 nM) (Da Pieve et al., 2009)]. An irrelevant Cy3 labeled aptamer was used as control for specificity of labeling (Cy3-control aptamer) (5'-ATGAGA TCTGAGTATAGTGTCCCGCAACCTTACACGAATGACGATTCTAGCGCGGCTGAT-3'). The mouse monoclonal immunoglobulin G (IgG) anti-MUC1 antibody (clone M4H2) was purchased from Abcam (K_d < 1.10^{-8} M). The rabbit anti-mouse IgG Fab2 Alexa555 and Alexa488-labeled secondary antibodies and calcein-acetoxymethyl ester (calcein-AM) were purchased from Molecular Probes (Life Technologies). Ethidium bromide (BET), D-glucose, magnesium chloride ($MgCl_2$), N-ethylmaleimide (NEM) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Washing buffer was prepared by adding 5 mM $MgCl_2$ and 4.5 g/L glucose into Dulbecco's phosphate buffer saline (DPBS) without calcium and magnesium (Gibco-Invitrogen). The binding buffer was prepared by adding 20 mg/mL BSA to the washing buffer.

Primary antibody direct labeling

Ten micrograms of M4H2 antibodies were directly labeled in 10- μ L reaction volume with the Thermo-Scientific Dy-Light 488 Amine-Reactive Dye (Thermo-Fischer Scientific) for 1 hour at room temperature. Volume was adjusted to 50 μ L prior to unbound dye removal on an Illustra MicroSpin G-50 column (GE Healthcare Life Science). The stoichiometry of labeling was determined spectrophotometrically at pH 8.5. The protein-bound dye concentrations were calculated using the following extinction coefficient: $\epsilon_{493\text{ nm}} = 70,000\text{ cm}^{-1}\text{ M}^{-1}$ for Alexa488. Antibody concentration after labeling was calculated using $\epsilon_{280\text{ nm}} = 210,000\text{ cm}^{-1}\text{ M}^{-1}$ with a previous correction from the absorption of the dye at this wavelength. The degree of labeling was 2.7 moles dye per mole of antibody.

Cell lines

The cell lines of human origin used in this study, human breast adenocarcinoma (MCF-7) were purchased from American Type Culture Collection. MCF-7 cells were grown and maintained in culture with Eagle's minimum essential medium (Gibco-Invitrogen) with 10% heat inactivated fetal bovine serum (Gibco), penicillin/streptomycin (100 units/mL; Gibco-Invitrogen), L-glutamine (0.58 mg/mL; Eurobio) and human insulin (0.01 mg/mL; Sigma). Cells were maintained at 37°C in a 5% CO_2 atmosphere. All experiments on plated cells were performed along the exponential growth phase. N-ethylmaleimide (NEM) was used at 5 mM during 30 minutes to block endocytosis.

Preparation of spheroids and labeling with aptamer or antibody

MCF-7 cells (2×10^4) were plated out in 96-well ultralow attachment plates (Corning Costar Sigma). After 5 days, spheroids were removed from the plate, placed in a 24-well plate and were rinsed twice in washing buffer (Gibot et al., 2013). For antibody labeling, 500 μ L of binding solution with 13 nM M4H2 MUC1 antibody were incubated with spheroids at room temperature for 30 minutes, washed twice with washing buffer then incubated with the rabbit anti-mouse secondary Alexa555 or Alexa488 antibody (1/2,000) for 30

minutes. Finally, spheroids were washed three times prior mounting in 1-mm-thick Cover Well™ imaging chamber (Grace Bio-Labs). For living and dead cells staining, spheroids were washed twice and then incubated with 1 μ g calcein-AM and 10 μ g BET for 30 minutes in 1 mL Dulbecco's PBS at 37°C. For labeling with aptamer, spheroids were washed twice, incubated in 500 μ L binding solution with 1 μ M (72 nt: 0.02 μ g/mL) folded (as described above) Cy3-MUC1 aptamer or Cy3-control aptamer for 5 minutes at room temperature, washed twice again, and mounted in a 1-mm-thick imaging chamber. Amounts of aptamers and antibodies used to obtain comparative fluorescence intensities were determined on 2D cell culture, where no hindrance to the access to MUC1 was present regardless of the ligand (Supplementary Fig. S1; Supplementary Data available online at www.liebertpub.com/nat).

Spheroids fluorescence macroscopy imaging

Fluorescence imaging was carried out on a fluorescence Macroscope (Leica Microsystems SA) with a 1 $\times$ objective, equipped with a Cool Snap HQ camera (Roper Scientific, Photometrics). Spheroids were imaged at $\times 2$ and $\times 4$ magnification, by fluorescence using appropriate filters (Alexa488: excitation filter, BP: 480/40 nm, emission filter, BP 527/30 nm (L5 set) ; Alexa555 and Cy3: excitation filter, BP: 560/40nm, emission filter, BP: 630/75 nm (EtmCH/TR set, Chroma Technology Corp.). Images were acquired and stored with Metavue software (Molecular Devices). Image analysis was performed off line using the Image J software (freely available from U.S. National Institutes of Health).

Spheroids two-photon microscopy imaging

Two-photon microscopy was carried out with a 7MP up-right microscope (Carl Zeiss S.A.S.). A Ti-Sapphire femto-second laser, Chameleon Ultra 2 (Coherent Inc.) was used as a laser source at 720 nm. For Alexa-555 and Cy3 observations, the emitted signal was directed into a non-descanned detector (barrier filter 565–610 nm). During experiments, all of the

images were acquired using an $\times 20/0.95$ NA immersion lens. Z-stacks were acquired on the TileScan mode 2×2 with $\times 0.7$ magnification and between 125 and 168 z-planes were taken, spaced 2 μ m apart. Each plane consists of an image of 1214 μ m by 1214 μ m (xy resolution: 0.59 μ m/pixel). Image analysis and multidimensional rendering was performed with Imaris Software (Bitplane AG).

Spheroids confocal microscopy imaging

Confocal microscopy was carried out on inverted LSM710 microscope (Carl Zeiss S.A.S.) equipped with the 40 $\times$ Zeiss objective (1.3 numerical aperture, oil immersion). Dylight488 and Cy3 were visualized using a 488-nm (emission filter: 497–578 nm) and 561-nm laser (emission filter: 565–641 nm) respectively. Eight-bit images were recorded with Zeiss LSM710 software (EMBL). The laser scan was unidirectional. Z-stacks were acquired with $\times 0.8$ and $\times 3$ magnifications spaced 2 μ m apart and 1 μ m apart respectively and between 34 and 51 z-planes were acquired. Each $\times 0.8$ magnification plane consists of an image of 265 μ m by 265 μ m (xy resolution: 0.26 μ m/pixel) and each $\times 3$ magnification plane consists of an image of 70.7 μ m by 70.7 μ m (xy resolution: 0.09 μ m/pixel).

Results

Both aptamer and antibody are effective to detect MUC1 on MCF-7 spheroids

To show whether aptamers may be used as specific and sensitive imaging probes for tumor detection, we analyzed the signal intensity for the MUC1 target expressed in MCF-7 spheroids when detected with Cy3-MUC1 aptamer (MW 22.4 kDa, $K_d = 0.135$ nM) relative to a specific anti-MUC1 antibody (M4H2) ($K_d < 10^{-8}$ nM). Cy3-MUC1 aptamer and anti-MUC1 antibody (revealed by an Alexa 555 secondary antibody) were incubated with spheroids and the labeling was analyzed by fluorescence macroscopy (Fig. 1). Cy3-MUC1 aptamer showed a diffuse signal spread all over the spheroid mass with a bell shaped distribution on the spheroid. This is the typical signature of a volumetric distribution of the tagged

FIG. 1. Imaging human breast adenocarcinoma (MCF-7) spheroids with mucin1 (MUC1)-specific labeling: Fluorescence macroscopy analysis of Cy3-MUC1 aptamer (A) and MUC1 antibody revealed with an Alexa555 secondary antibody (C) showed that both probes labeled MCF-7 spheroids. No detectable staining was observed with either the irrelevant Cy3-control aptamer (B) or the Alexa555 secondary antibody (D). Spheroids were incubated with 1 μ M of aptamers for 5 minutes or with 13 nM of MUC1 antibody for 30 minutes. Scale bars = 200 μ m (C); 600 μ m (B); 1200 μ m (A and D).

FIG. 2. Specific labeling by MUC1 aptamer on MCF-7 spheroids. Z-confocal projections of MCF-7 spheroids labeled with the Cy3-MUC1 aptamer (A), an irrelevant Cy3-control aptamer (B), and a live-dead cell staining with calcein-acetoxymethyl ester and ethidium bromide (C) were performed. The Cy3-MUC1 aptamer exhibits a diffuse labeling all over the spheroid with some focused points (A). The same focused points were observed with an irrelevant Cy3-control aptamer but without diffuse labeling (B). Focused points are due to dead cells as shown by the staining with ethidium bromide (C). Spheroids were incubated with 1 μ M of aptamers for 5 minutes. Scale bars = 200 μ m.

aptamer accumulated essentially inside the spheroid (Fig. 1A). This detection is illustrative of the specificity of our probe as shown by the nonsignificant weak signal observed in the case of the Cy3-control aptamer that was randomly designed (Fig. 1B). The chemical modification associated to the covalent tagging of the fluorophore is not affecting the selectivity obtained by SELEX. Anti-MUC1 antibody showed a fluorescent signal restricted to the cells that were localized at the periphery of the spheroid (Fig. 1C). No labeling was obtained when the secondary Alexa555 antibody was used alone (Fig. 1D) or when no probe was used (Supplementary Fig. S2D).

Image analysis of the labeling by MUC1 aptamer and MUC1 antibody showed that the global fluorescence associated with the spheroid was within the same range for the two probes. The fluorescent yield was indeed 2.5 times higher for the antibody (see Supplementary Data). This comparison was possible because the excitation and the emission of the two fluorophore (Cy3 and Alexa 555) were practically in the same wavelength range. The same results were obtained with all spheroids that were analyzed (Supplementary Fig. 2A, C). To conclude, aptamers were at least as effective as antibodies to image the spheroid.

MUC1 aptamer is specific to detect MUC1 on MCF-7 spheroids

To assess the specificity of labeling with Cy3-MUC1 aptamer, we compared the labeling of spheroids with the Cy3-MUC1 aptamer (Fig. 2A) and the irrelevant Cy3-control aptamer (Fig. 2B) using fluorescence microscopy. Only a weak nonspecific labeling was observed inside spheroid with discrete localizations (Fig. 2B) compared to the homogeneous pattern obtained with Cy3-MUC1 aptamer (Fig. 2A). It clearly indicated that the specific aptamer labeling had two origins: a nonspecific trapping and a specific interaction with MUC1 target. Dead or necrotic cells were present in the center of spheroids (Wenning and Murphy, 1999), and we hypothesized that the nonspecific labeling observed was due to interaction with genomic DNA released from dead cells. This hypothesis was based on experiments realized on fixed MCF-7 cells, which clearly showed a nuclear labeling with any aptamer (Supplementary Fig. S3). The proportion of living cells and free genomic DNA in our spheroids were checked by using calcein-AM and BET (Fig. 2C). These results confirmed that living cells did not show any BET

FIG. 3. A same z-plane confocal exploration shows a penetrating labeling with MUC1-aptamer and a peripheral MUC1-antibody labeling in MCF-7 spheroid. The same spheroid was labeled simultaneously with Cy3-MUC1 aptamer (A) and anti-MUC1 antibody revealed with an Alexa488 secondary antibody (B). The merge image (C) showed colabeling in periphery and a unique aptamer labeling inside the spheroid. Spheroids were incubated with 1 μ M of aptamers for 5 minutes or with 13 nM of MUC1 antibody for 30 minutes. Scale bars = 200 μ m.

staining and that the focused spots corresponded to nonspecific labeling. Therefore, the diffuse labeling observed all over the spheroid (Fig. 2A) was due to the specific detection of MUC1 by the Cy3-MUC1 aptamer.

MUC1 aptamer penetrates inside MCF-7 spheroids

Internal labeling of spheroids by MUC1 aptamers was further investigated by confocal microscopy. Spheroids were simultaneously incubated with Cy3-MUC1 aptamer (Fig. 3A) and anti-MUC1 antibody revealed with an Alexa488 secondary antibody (Fig. 3B). Confocal microscopy allowed a deeper definition inside the spheroid than did fluorescence macroscopy. On the central z-plane image, most of the surface was labeled with Cy3-MUC1 aptamer (Fig. 3A). By contrast, anti-MUC1 antibody only showed a defined labeling of cells located on the periphery of spheroids. No evidence of internal labeling was detected with anti-MUC1 antibody (Fig. 3B).

The colocalization of the aptamer and the antibody showed that the cells located at the surface of the spheroid were labeled by the two probes (Fig. 3C). We checked a possible binding competition for the membrane MUC1 antigen between aptamer and antibody and verified that the Alexa488

fluorescence signal was not affected by the cells' absorption in spheroid. Indeed, due to the different wavelength ranges of fluorophores, higher scattering and absorption may contribute to the decrease of the emitted signal of fluorescence from the cells located inside the spheroid. Therefore, we replaced the labeling of the secondary antibody by Alexa555, which had similar excitation/emission wavelengths to Cy3. Cy3-MUC1 aptamer and anti-MUC1 antibody revealed by Alexa555 secondary antibody were added on separated spheroids (Fig. 4A, B). The orthogonal cross sections showed that the labeling obtained with Cy3-MUC1 aptamer and anti-MUC1 antibody revealed by Alexa555 secondary antibody were similar to previous experiments using Alexa488 (Fig. 3B). Analysis with Cy3-MUC1 aptamer labeling showed the uniform and deep labeling of cells inside the spheroid (Fig. 4C). On the other hand, anti-MUC1 antibody again showed exclusively peripheral cell labeling and was not able to bind cells inside the spheroid (Fig. 4D). The comparison between Fig. 4C and 4D on the z-plane cross section clearly confirmed that aptamer had a deeper penetration inside spheroids than antibody, exhibiting a more homogeneous labeling on 3D tumor models.

In all previous experiments, the anti-MUC1 antibody was revealed with a labeled secondary antibody. To verify that the

FIG. 4. Z-confocal projections and orthogonal cross sections exploration of spheroid penetrating skills of MUC1-aptamer and MUC1-antibody: z-confocal projection images with Cy3-MUC1 aptamer (A) and anti-MUC1 antibody revealed with Alexa555 secondary antibody (B). The orthogonal cross-sections reveal a good penetration of the Cy3-MUC 1 aptamer (C) and an exclusive peripheral labeling of the anti-MUC1 antibody (D). Spheroids were incubated with 1 μ M of aptamers for 5 minutes or with 13 nM of MUC1 antibody for 30 minutes. Scale bars = 200 μ m.

FIG. 5. Penetration depth and cellular localization of Cy3-MUC1 aptamer and primary labeled Dylight 488-MUC1 antibody on MCF-7 spheroids. Images corresponded to the overlay of bright field images and fluorescence signals. (A, B) The Cy3-MUC1 aptamer penetrated between the spheroid cells and was internalized. Anti-MUC1 antibody was directly labeled with Dylight 488. (C) Anti-MUC1 antibody did not penetrate inside the spheroid and was not internalized by cells. Spheroids were incubated with 1 μ M of aptamers during 5 minutes or with 13 nM of MUC1 antibody for 30 minutes. Scale bars = 50 μ m.

poor penetration of anti-MUC1 antibody in the spheroid was not due to a limited accessibility of the secondary antibody, we directly labeled the anti-MUC1 antibody with Dylight 488. MCF-7 spheroids were incubated with Cy3-MUC1 aptamer (Fig. 5A, B) or Dylight 488 anti-MUC1 antibody (Fig. 5C) and then imaged by overlay of phase contrast and fluorescence confocal microscopy. The data confirmed that the antibody only labeled the cells located on the periphery of the spheroid (Fig. 5C) and the absence of internal spheroid labeling. This might be due to the inaccessibility of the primary antibody to its membrane target inside the spheroid. We propose that this is linked to the presence of the extracellular matrix network that prevents the access for large molecules such as antibodies while leaving a free diffusion to the smaller aptamers.

Aptamers are internalized into the cells of spheroids

Spheroid cells were almost entirely labeled by the Cy3-MUC1 aptamer (Fig. 5A) and the enlargement showed labeling of intracellular vesicles (Fig. 5B), as previously reported (Chang et al., 2011). Moreover, in our conditions, antibody was not internalized into cells and only surfaces of detected cells were labeled. These results demonstrated that in this spheroid model, aptamers, besides their deeper penetration, were internalized inside cell vesicles. To confirm this result, MCF-7 cells were treated with NEM, an inhibitor of endocytosis (Sandvig et al., 2000), before incubation with MUC1 aptamer (Supplementary Fig. S4). Cells treated with NEM lacked the internal labeling observed without NEM incubation.

Discussion

In this study, we used MCF-7 spheroids as a tumor model to explore the ability of MUC1 aptamer to detect cancer cells and therefore be used as an imaging probe for diagnostic and intraoperative detection of cancer cells. Our major findings showed that aptamers could advantageously replace antibodies. They possess desirable attributes for molecular imaging because they penetrate deeply inside the core of the spheroid, whereas anti-MUC1 antibody only interacts with the cells located on the surface of the spheroid.

Spheroids are 3D cell culture models that have shown their importance in the behavior of biological system culture especially in cancer research. For example, many studies have demonstrated elevated levels of drug resistance in spheroids compared with cell monolayers (Hoffman, 1993). The spheroid model appears to be the best current system for an *ex vivo* model of tumors.

The use of molecular probes that specifically discriminate cancer cells from healthy ones is a powerful tool and represents a considerable progress in tumor diagnosis, as it allows tumors to be precisely circumscribed during intraoperative intervention. Antibodies that recognize a specific biomarker like CEA or epithelial growth factor receptor (EGFR) were used to visualize cancer cells during surgery (Terwisscha van Scheltinga et al., 2011). Up to now, antibodies were considered as the most specific probes for tumor imaging due to their high affinity and specificity (Gao et al., 2004; Cohen and Margel, 2012; Kaur et al., 2012). However, antibodies present two major restrictions for *in vivo* tumor imaging: (1) their immunogenicity, and (2) the difficulty to obtain the chemical modifications needed for fluorescent labeling but also for *in vivo* stability (Worn and Pluckthun, 2001; Jin et al., 2013). Nucleic acid aptamers have emerged as new targeting probes with excellent potential for imaging applications (Cerchia and de Franciscis, 2010; Ruigrok et al., 2011; Zhu et al., 2012). They considerably decrease side effects because of their lack of immunogenicity, and they can easily be chemically modified with, for example, the addition of fluorescent conjugates that allow the specific detection of the target for diagnosis or during surgery.

Here we compared the well-characterized MUC1 aptamer with anti-MUC1 antibody by different fluorescence imaging approaches using spheroids. In our model, cells were detected by MUC1 aptamer all over the spheroid mass, whereas anti-MUC1 antibody showed a labeling limited to the periphery of the spheroid. *In vivo*, enhanced permeability and retention effect (EPR effect) is an important property of solid tumors and clearly increases the penetration of macromolecules (or nanoparticles, liposomes) inside tumors (Maeda, 2001). The leaky vasculature associated with EPR effect of these tumors enhances the spread of macromolecules inside the tumors.

Our results showed that aptamers were more efficient to spread into spheroids than antibodies. The diffuse signal obtained from the aptamers demonstrated their capacity to uniformly infiltrate the tumor mass.

Fluorescence imaging gave convincing results concerning the penetration capacity of aptamers versus antibodies in our spheroid model. As described in the literature (Shigdar et al., 2013), the labeling with antibodies was restricted to the external layer of the spheroid, but their images did not allow an estimation of the depth of aptamer penetration inside the spheroid. Biphotonic microscopy led us to obtain more precise images on this aspect. Z-plane cross sections permitted us to investigate the penetration depth of both aptamers and antibodies inside the spheroid. These observations clearly demonstrated the poor penetration capacity of antibodies and their accumulation on the external layer of the spheroid. The fact that the detection was done with a secondary antibody was addressed and the apparent limited penetration of the primary antibody was also observed when it was directly conjugated with Dylight 488, since only external spheroid layers are labeled. Thus, the information confirmed that the antibody penetration was limited to a few micrometers in depth. In contrast, aptamers showed a continuous labeling across the spheroid.

The observation that antibodies do not penetrate into tumor spheroids was already well-documented (Wenning and Murphy, 1999; Graff and Wittrup, 2003; Thurber and Wittrup, 2008; Shigdar et al., 2013). This limited diffusion was also observed for antibody fragments like scFv (Sutherland et al., 1987) and was mainly explained by the presence of tight junction complexes between adjacent cells that represent a significant barrier to penetration by these proteins. It appears from our results that nucleic acids may bypass these barriers to access the center of the spheroid (Thurber et al., 2007).

This property suggests that aptamers could not only be useful for imaging, but also as delivery tools. Recently, aptamers coupled with toxin (Chu et al., 2006), small interfering RNA (McNamara et al., 2006; Dassie et al., 2009), micro RNA (Dai et al., 2012; Liu et al., 2012) or functionalized nanoparticles (Farokhzad et al., 2006; Yu et al., 2011; Chen et al., 2012) have been shown to be effective in drug-targeting to cancer cells *in vitro* and *in vivo* (Kanwar et al., 2011; Wang and Li, 2011; Li et al., 2013).

Interestingly, the intracellular labeling showed that aptamers were rapidly internalized by intracellular vesicles. We have shown that this labeling was specific for MUC1 aptamer and that an irrelevant aptamer detected only the few dead cells present in spheroids. The same vesicular labeling with anti-MUC1 aptamer conjugated nanoparticles as carrier for doxorubicine was shown to colocalize with late endosomes/lysosomes and to deliver the chemotherapeutic drugs inside the cytoplasm (Chang et al., 2011). This rapid and efficient degradation of the probe in the endocytosis pathway represents a strong possibility to deliver amphipathic drugs inside tumor cells. Recently, results with CD133 aptamer have shown that these probes remain sufficiently long within the tumor mass to efficiently deliver chemotherapeutic drugs (Shigdar et al., 2013).

Using a high resolution imaging approach, our work clearly supports the advantage of small targeting probes like aptamers to efficiently penetrate inside solid tumors.

Conclusion

Cy3-MUC1 aptamer was able to label cancer cells in a tumor spheroid model. Furthermore, the aptamer penetrated deep into the tumor mass, where it was efficiently internalized inside cell vesicles once the biomarker was detected. This was in contrast to anti-MUC1 antibody, which was confined to the outside of the spheroid.

The small size, the easy chemical modifications and the lack of immunogenicity of aptamers are transforming these molecules as powerful tools that can effectively substitute for antibodies in tumor detection for diagnostics as well as intraoperative image-guided surgery. It is now needed to confirm our results with *in vivo* experiments and to further explore the potential of aptamers as probes to deliver drugs deep inside solid tumors and as theranostic tools.

Acknowledgments

Financial support for this project was provided by the RITC Foundation of Toulouse (Fondation Recherche et Innovation Thérapeutique en Cancérologie). This project was approved by the Pole de Compétitivité Cancer-Bio-Santé. This work has been performed in collaboration with the Toulouse Réseau Imagerie core of Institut de Pharmacologie et de Biologie Structurale (IPBS) facility (Genotoul, Toulouse, France), which is supported by the Association Recherche Cancer (no. 5585, no. 8505), Région Midi-Pyrénées (CPER), and Grand Toulouse cluster. We thank Michael Shirber for carefully reading the manuscript.

Author Disclosure Statement

No competing financial interests exist.

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Received for publication August 2, 2013; accepted after revision January 2, 2014.