Proceedings of the 26th Annual International Conference of the IEEE EMBS
San Francisco, CA, USA • September 1-5, 2004

Molecular Imaging of Carcinogenesis with Immuno-Targeted Nanoparticles

K. Sokolov[1], J. Aaron[2], S. Kumar[2], V. Mack[2], T. Collier[2], L. Coghlan[3], A. Gillenwater[4], K. Adler Storthz[5], M. Follen[6], R. Richards Kortum[2]

[1]Department of Imaging Physics, UT M.D. Anderson Cancer Center, Houston, TX, USA
[2]Department of Biomedical Engineering, University of Texas, Austin, TX, USA
[3]The University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX, USA
[4]Department of Head and Neck Surgery, UT M.D. Anderson Cancer Center, Houston, TX, USA
[5]Department of Molecular and Cellular Oncology, UT M.D. Anderson Cancer Center, Houston, TX, USA
[6]Biomedical Engineering Center and Department of Gynecologic Oncology, UT M.D. Anderson Cancer Center, Houston, TX, USA

Molecular characterization of cancer could have important clinical benefits such as earlier cancer detection based on molecular characterization, the ability to predict the risk of cancer progression, real time margin detection, the ability to rationally select molecular therapy and to monitor response to the therapy. We present a new class of molecular specific contrast agents for optical imaging of carcinogenesis *in vivo* - gold nanoparticles conjugated with monoclonal antibodies specific for cancer biomarkers.

Keywords—**molecular imaging, gold nanoparticles, cancer imaging**

I. Introduction

Gold nanoparticles can resonantly scatter visible light that provides an opportunity for optical interrogation using reflectance spectroscopic and imaging modalities [1-3]. We demonstrate molecular imaging of three cancer related biomarkers which are associated with many cancers, including cancers of the oral cavity and the cervix: the epidermal growth factor receptor (EGFR), matrix metallopreinases (MMPs), and E7 oncoprotein associated with human papillomavirus (HPV). Using progressively more complex biological models of cancer, ranging from cancer cells in suspension to in vivo animal models, we have demonstrated that the optical contrast agents based on gold nanoparticles have the potential to provide sensitive tool to image the molecular changes associated with carcinogenesis. Using cancer cells in suspension and in 3-D tissue culture, we demonstrated labeling of cancer related biomarkers up to a depth of 500 microns using simple penetration enhancers. Fresh tissue slices from normal and precancerous human oral mucosa and cervix show overexpression of EGFR in neoplasia detected optically using these contrast agents. Finally, we present data from animal models of cancer, the DMBA-hamster cheek pouch model and subcutaneous tumor in nude mouse, which illustrate that the contrast agents can be used in vivo to image EGFR overexpression in neoplasia.

II. Methodology

Gold nanoparticles were prepared from chloauric acid ($HAuCl_4$) by using sodium citrate as a reducing agent [4]. Conjugates of gold nanoparticles with monoclonal antibodies (mAb) were prepared using procedure described in [5] which was slightly modified. Briefly, concentrated HEPES buffer was added to a colloidal solution up to final concentration of 10 mM. The pH of the buffer was adjusted to the isoelectric point (point of zero charge) of a protein. Proteins were reconstituted in the same buffer and mixed with the metal colloidal solution at the final concentration of 50 μg/ml. At the isoelectric point proteins form very stable and irreversible complex with metal nanoparticles. After a short incubation time the protein/metal particles conjugates were washed in PBS and resuspended in PBS buffer containing 0.2 mg/ml polyethylene glycol and 5 mg/ml BSA.

Cells were grown inside tissue culture flasks covered with collagen type I (Roche) in DMEM plus 5% FBS at 37°C under 5% CO_2. Cells were harvested using 1 mg/mL collagenase (Roche) in phosphate buffered saline at 37°C for approximately 20 minutes, or until the collagen substrate was entirely disassociated, and were washed in DMEM. The cell suspension was labeled with gold conjugate at room temperature for *ca.* 30 minutes on a shaker to prevent sedimentation. The labeled cells were placed on top of a microscope slide coated with gelatin to eliminate background scattering from the glass substrate during reflectance imaging.

To prepare tissue constructs a suspension of epithelial cells was spun down and a very small amount of buffered collagen type I solution (3 mg/ml) was added to the pellet. The mixture was transferred to 6.5 mm transwells and allowed to gel at 37°C for 20 minutes. The volume of the mixture was adjusted to form gels with thickness between 400 and 600 μm. The gel with embedded cells was kept in DMEM culture medium plus 5% FBS for 24-48 hours. During this time the cells continued to grow resulting in formation of a highly dense structure consisting of multiple layers of epithelial cells. The contrast agents were added on top of the tissue phantoms in 10% polyvinyl pyrrolidone (PVP) or 10% DMSO solution in PBS or in pure PBS. After incubation for ca. 30 minutes at room temperature the

0-7803-8439-3/04/$20.00©2004 IEEE

phantoms were transversely sectioned with a Krumdieck tissue slicer and the sections were imaged using Zeiss Leica inverted laser scanning confocal microscope.

Colposcopically normal and abnormal cervical biopsies were obtained, with written consent, from women seen in the University of Texas M.D. Anderson Cancer Center Colposcopy Clinic. Biopsies were immediately placed in chilled (4°C) culture medium (Dilbecco Modified Eagle Medium without phenol red), and then embedded in 4% agarose up to the epithelial layer of the biopsies. The contrast agents were added on top of the epithelium of the embedded biopsies in 10% DMSO solution in PBS. After incubation for ca. 1 hour at room temperature the biopsies were transversely sectioned with a Krumdieck tissue slicer and the sections were imaged using Zeiss Leica inverted laser scanning confocal microscope.

Subcutaneous nude mouse tumor model. In this model cervical cancer SiHa cells were injected in mammary fat pad of nude immunodeficient mouse. After one to two weeks a tumor growths at the site of the injection. For optical imaging experiments the tumor was exposed in skin flap and anti-EGFR gold nanoparticles applied topically in 10% DMSO.

Syrian hamster cheek pouch model. In this model the tumor is induced using chronic treatments with the carcinogen dimethylbenz[α]anthracene (DMBA) in the cheek pouch. Histologically, the 16-week treatment protocol pushes the epithelial lining of the cheek pouch through stages of inflammation, hyperplasia, dysplasia, and both benign and malignant tumor formation. Epithelial hyperplasia develops after only a few treatments with DMBA. DMBA treated and control animals were anesthetized and imaged using reflectance confocal microscope, prior to and after application of anti-EGFR gold conjugates in 10% DMSO in saline PBS buffer.

Confocal microscopy. The series of through focus confocal images were acquired using Zeiss Leica inverted epi-fluorescence/reflectance laser scanning confocal microscope with a 40X oil immersion objective or a 10X objective. The excitation was provided by a Kr/Ar mixed gas laser.

III. Results and Discussion

A. Imaging of EGFR

Figure 1 shows bright-field and confocal reflectance images of SiHa cells labeled with gold particles/anti-EGFR monoclonal antibodies conjugates. Bright rings around the cellular cytoplasmic membrane of the SiHa cells can be clearly seen. This labeling pattern is consistent with the fact that the monoclonal antibodies have molecular specificity to the extracellular domain of EGFR. The intensity of light scattering from the labeled SiHa cells is ca. 50 times higher

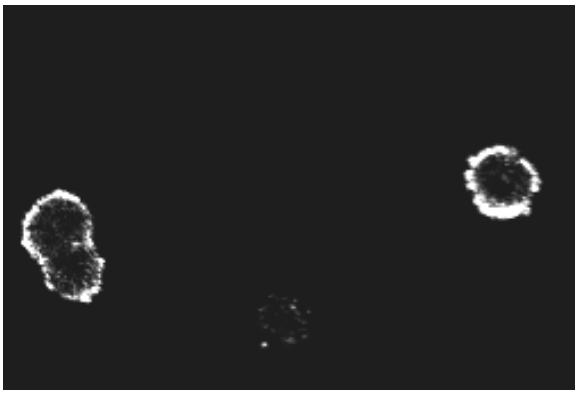

Fig. 1. Confocal reflectance images of SiHa cells labeled with anti-EGFR antibodies/gold nanoparticles conjugates.

than from unlabeled cells. Therefore unlabeled cells cannot be resolved on the dark background. No labeling was observed when gold conjugates with nonspecific mouse monoclonal IgG antibodies were added to the cells. Light scattering from the labeled cells is so strong that it can be easily observed using low magnification optics and an inexpensive light source such as a laser pointer [2].

In vivo application of these contrast agents depends on the ability to deliver the agents throughout the epithelium in the organ site of interest. Pre-cancers of squamous epithelium originate at the basal layer, which can be located 300-500 μm beneath the tissue surface. Therefore, to image the earliest molecular changes associated with carcinogenesis the gold bioconjugates have to be delivered throughout the whole epithelium. First, we used engineered tissue constructs to demonstrate that penetration enhancers used for topical drug delivery, such as polyvinyl pyrrolidone (PVP) and DMSO, can be used to deliver the gold nanoparticles throughout the epithelial thickness [2]. Subsequently, we extended our work using anti-EGFR gold nanoparticle conjugates to label organ cultures of normal and neoplastic cervical tissue. Gold bioconjugates with anti-EGFR monoclonal antibodies in 10% DMSO in PBS saline solution were applied on top of cervical and oral cavity biopsies at room temperature. Then the biopsies were washed in PBS and 200-micron transverse sections were prepared and then imaged with reflectance based confocal microscopy. The bright "honey-comb" like structure of labeled cellular cytoplasm membranes of closely spaced cells can be easily seen in clinically abnormal samples of cervical biopsies obtained using confocal reflectance microscope (Fig. 2).

We also demonstrated *in vivo* application of the anti-EGFR gold bioconjugates in the subcutaneous tumor model in nude mice. The tumors were exposed in a skin flap and the contrast agents were applied in 10% DMSO in PBS saline. After incubation, 200 micron transverse sections of

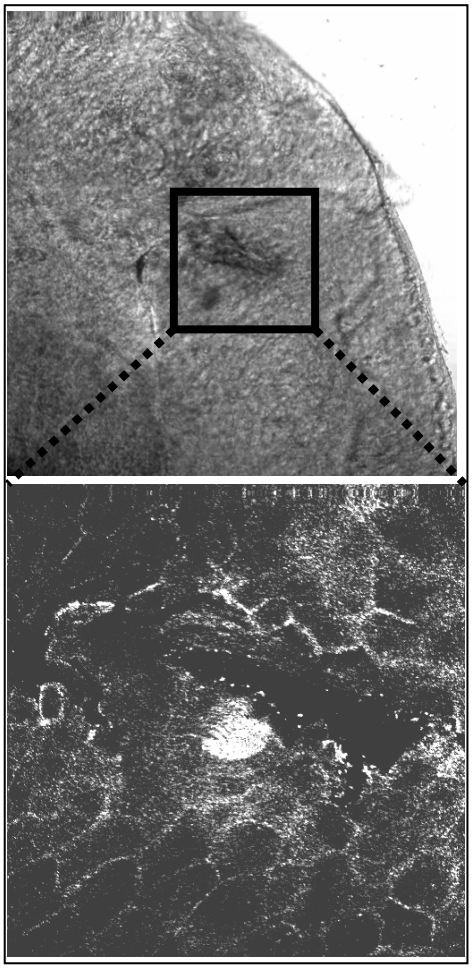

Fig. 2. Abnormal cervical biopsy after topical application of anti-EGFR gold conjugates in 10% DMSO in PBS saline. Top image: bright-field transmittance image obtained with 10X objective. Note dark region due to absorption of the transmitted light by the metal nanoparticles. Lower image: confocal reflectance of the region marked with black square in the transmittance image (40X objective, 647 nm excitation).

the tumor were obtained and imaged using confocal reflectance microscopy. The characteristic "honey-comb" pattern of the labeled cancer cells were observed in the tumors exposed to the anti-EGFR gold conjugates.

In another set of experiments we used the well characterized hamster cheek pouch carcinogenesis model. The contrast agents were applied *in vivo* in 10% DMSO in saline PBS buffer to normal and treated animals and then the animals were imaged *in vivo* using reflectance confocal microscope. Biopsies were obtained following imaging and analyzed for EGFR expression and tissue morphology. EGFR specific labeling throughout the epithelium was observed in confocal reflectance images *in vivo* of DMBA

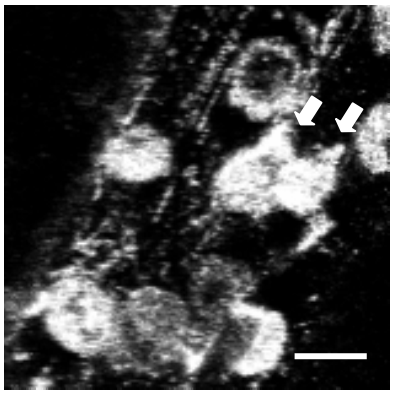

Fig. 3. Confocal reflectance image of SiHa cells on collagen I labeled with anti-MMP-9 /gold conjugates. 40X objective, 647 nm excitation. The scale bar *ca.* 10 micron.

treated hamster but not in the control. This result was confirmed using immunohistochemical staining of cheek pouch biopsies from treated and normal hamster.

Therefore, we demonstrated that gold bioconjugates with monoclonal antibodies can be used for molecular specific imaging *in vivo* with high optical contrast between tissue overexpressing EGFR and tissue with low level of EGFR expression.

B. Imaging of MMPs

We used gold particles conjugated with monoclonal antibodies to MMP-2 and MMP-9; enzymes frequently overexpressed during development and progression of cancer. We labeled cervical epithelial cancer cells (SiHa) grown on two different substrates: a pure collagen I gel and a collagen I gel in the presence of 5% gelatin. SiHa cells were placed on a substrate and allowed to grow for 5-24 hrs in DMEM with 5% FBS at 37°C and 5% CO2, and then antibody-gold conjugates were applied in PBS for 20-30 minutes under sterile conditions. The sample was then imaged using a Leica laser scanning confocal reflectance/fluorescence microscope without an intermediate washing step. Imaging without washing of unbound metal nanoparticles is possible due to significantly higher scattering cross-section of closely spaced nanoparticles in comparison to individual unbound conjugates. This property allows selective imaging of areas where there is significant up-regulation of a particular target [2,3].

Labeling with MMP 2 specific contrast agents was mostly confined to the plasma membrane of the cells whereas significant labeling of cellular cytoplasm was observed with MMP-9 specific agents (Fig. 3). Surrounding some of the cells labeled with MMP 2 specific contrast agents were fibrous structures suggestive of collagen fibers with bound MMP 2 molecules. The MMP 2 labeling was significantly decreased when SiHa cells were grown on collagen I in the presence of gelatin. We also observed

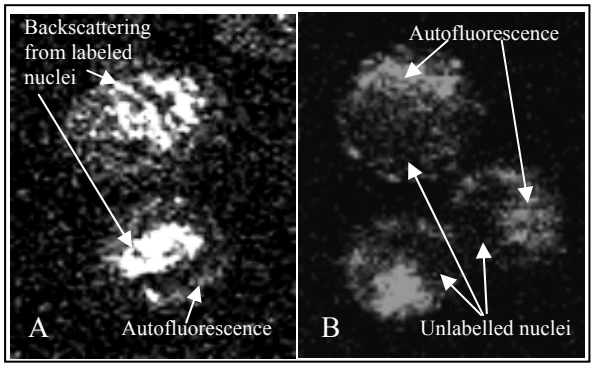

Fig. 4. Co-localized fluorescence (grey) and reflectance (white) laser scanning confocal microscope images of Caski cells incubated with anti-E7 nanoparticle conjugates (A) and nonspecific IgG nanoparticle conjugates (B) in 10% PVP in PBS. Strong backscattering from nanoparticles is seen in the nuclei of the Caski cells incubated with anti-E7 conjugates. Reflectance images were obtained with 40X objective and 647 nm excitation; fluorescence – 40X objective, 488 nm excitation and 515 nm long band-pass emission filter.

strong labeling of collagen fibers located along clusters of cells labeled with MMP 9 specific conjugates (Fig. 3).

Labeling of MMPs with gold nanoparticles produces sharp optical images of cancer cells. We suggest that cytoplasmic labeling is associated with internalization (through endocytosis) of labeled MMP-9 molecules from the plasma membrane [6]. Strong labeling of collagen fibers located along clusters of cells was previously observed using fluorescent labeled antibodies in fixed 3D tissue cultures of cells inside collagen I matrix [7]. It was attributed to membrane deposits which are shed by migrating cells along their tracks in collagen matrix. These deposits contain a variety of plasma membrane proteins including MMPs. Our results support and extend these observations.

C. Imaging of E7 oncoprotein

One of our goals is to develop delivery formulations and approaches for specific labeling of intracellular targets associated with carcinogenesis. As part of this effort we are developing contrast agents for HPV induced carcinogenesis. One of the key features of the HPV induced cervical carcinogenesis is expression of E7 oncoprotein which is an intracellular target, predominately located in the nucleus [8].

In Fig. 4, we demonstrate our preliminary data obtained by labeling Caski cervical cancer cells which have high level of expression of E7 protein. The cells were incubated in the suspension of gold nanoparticles conjugated with anti-E7 monoclonal antibodies in 10% PVP in PBS saline. After the incubation the cells were washed in PBS and reflectance confocal images were obtained to assess location of the contrast agents. In addition, fluorescence confocal images were measured to assess cellular autofluorescence which is limited to the cytoplasm. Fig. 4 shows that the backscattering signal is localized in the dark central area of the cells which corresponds to the nucleus. This result is consistent with the location of the E7 protein. Currently we are exploring mechanisms of intracellular delivery of the contrast agents inside living cells in order to optimize imaging of intracellular targets using gold bioconjugates.

V. Conclusion

We presented a new class of molecular specific contrast agents based on gold nanoparticles which provide bright optical signal and contrast enhancement in reflectance imaging modalities. The contrast agents can be used for micro-anatomic and molecular *in-vivo* imaging of tissue pathology such as cancer. We demonstrated an approach based on the combination of the contrast agents with tissue permeation agents that may overcome a critical barrier in bringing these optical contrast agents to *in-vivo* clinical use. The proposed contrast agents can significantly impact current clinical practice providing the ability for rapid and non-invasive screening and detection of pre-cancers, and monitoring of cancer therapy all based on molecular signatures of carcinogenesis.

References

[1] S. Schultz, et al., "Single-target molecule detection with nonbleaching multicolor optical immunolabels," *PNAS*, vol. 97, no. 3, pp. 996-1001, 2000.

[2] K. Sokolov, et al., "Gold Nanoparticles as Molecular Specific Contrast Agents for Real Time Vital Optical Imaging," *Cancer Research*, vol. 63, pp. 1999-2004, 2003.

[3] K. Sokolov, J. Aaron, B. Hsu, D. Nida, A. Gillenwater, M. Follen, C. MacAulay, K. Adler-Storthz, B. Korgel, M. Descour, R. Pasqualini, W. Arap, W. Lam, R. Richards-Kortum, "Optical systems for in vivo molecular imaging of cancer," *TCRT*, vol. 2, no. 6, pp. 491-504, 2003.

[4] G. Frens, "Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions," *Nature Physical Science*, vol. 241, pp. 20-2, 1973.

[5] W.D. Geoghegan and G.A. Ackerman, "Adsorption of Horseradish Peroxidase, Ovomucoid and Anti-Immunoglobulin to Colloidal Gold for the Indirect Detection of Concanavalin A, Wheat Germ Agglutination and Goat Anti-Human immunoglobulin G on Cell Surface at the Electron Microscope Level: a New Method, Theory and Application," *J of Histochemistry and Cytochemistry*, vol. 25, pp. 1187-1200, 1977.

[6] E. Hahn-Dantona, et al., "The low density lipoprotein receptor-related protein modulates levels of matrix metalloproteinase 9 (MMP-9) by mediating its cellular catabolism," *The Journal of Biological Chemistry*, vol. 276, no. 18, pp. 15498-503, 2001.

[7] P. Friedl and E.-B. Brocker, "The biology of cell locomotion within three-dimensional extracellular matrix." *Cellular and Molecular Life Sciences*, vol. 57, no. 1, pp. 41-64, 2000.

[8] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, J. Watson, *Molecular Biology of the Cell*, Garland Publishing, New York, 1994.