9 Imaging of Proteases for Tumor Detection and Differentiation

C. Bremer

9.1 Introduction 159
9.2 Light for Molecular Imaging 160
9.3 Optical Contrast Agents 161
9.3.1 Oncological Imaging 163
9.4 Nononcological Imaging 165
9.5 Potential Clinical Applications 166
References 168

9.1 Introduction

Major challenges for the molecular imaging community are (a) defining diagnostically relevant targets and (b) developing suitable imaging methods to noninvasively depict those targets. Various molecular substrates such as DNA, RNA, or proteins could serve as potential imaging targets. However, targeting intracellular structures in vivo is a major pharmacological challenge since several delivery barriers have to be overcome. Moreover, there is a natural "signal amplification" in the transcription and translation process resulting in several copies of RNA from one DNA template and again multiple protein copies of the mRNA. Therefore "downstream imaging" (i.e., molecular imaging on the protein level) should be the most promising approach to detecting molecular structures in an intact organism.

Compared to conventional imaging approaches even targeting whole cells, which measure 5–20 µm in diameter in size, is an ambitious goal. However, looking at protein structures is an even more challenging task and therefore requires efficient contrasting strategies with excellent signal-to-noise ratios (SNR). Nuclear imaging modalities have been the mainstay for in vivo probing of molecular substrates since radioactive tracers are employed without any natural background signal and therefore high SNR can be achieved. In contrast, detecting molecular structures by magnetic resonance imaging (MRI) is a very challenging task as protons are omnipresent within the body because approximately 65% of the body consists of water, contributing to a high background signal. More recently, very promising approaches have been undertaken to exploit "smart" MR-contrast agents as well as carbon or fluorine compounds serving as specific molecular contrast agents in vivo.

9.2 Light for Molecular Imaging

Using light for in vivo imaging is an emerging imaging modality which offers some unique advantages for molecular in vivo imaging:

1. Different optical techniques have already been used for many years in the molecular biology community to probe tissue samples or cells (such as the use of fluorescent proteins, fluorescently labeled antibodies, etc.) and can be adapted for in vivo applications.
2. In the near infrared range (NIR) very little tissue auto-fluorescence can be observed offering excellent signal-to-noise ratios (SNR) comparable to conventional nuclear imaging techniques. Besides offering high SNR, light in the NIR can traverse tissue very efficiently as the absorption by water and hemoglobin is relatively low in this spectrum ("diagnostic window"; Ntziachristos and Weissleder 2001).
3. Functional paradigms can be applied for optical contrast agents (such as fluorescence resonance energy transfer or bioluminescence) in order to detect specific target interactions in vivo (such as enzyme activity).

9.3 Optical Contrast Agents

Various strategies can be pursued to obtain optical information from the tissue ranging from nonspecific perfusion contrast agents to targeted or “smart” optical probes (Fig. 1).

While nonspecific perfusion agents such as indocyanine green (ICG) can only depict tumor physiology such as perfusion, vessel permeability, or tissue blood volume, molecular information can not be obtained using this approach.

Targeted contrast agents, however, are linked to affinity ligands imparting molecular specificity to the optical probe. Ideally, these

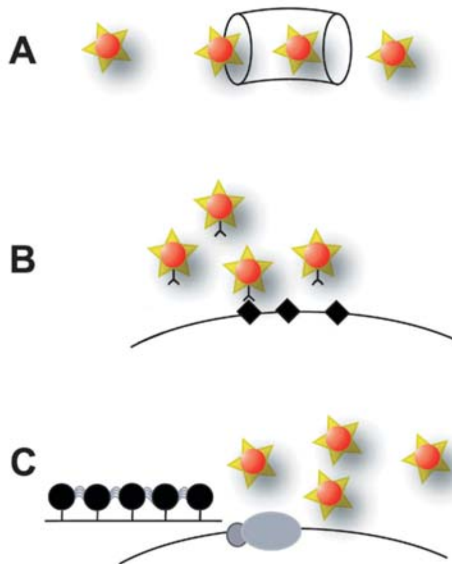

Fig. 1 A–C. Design of different optical contrast agents. Nonspecific fluorochromes such as indocyanine green (**A**) show perfusion and/or permeability properties of the tissue. Targeted probes (**B**) bind via specific ligands to protein structures on the cell surface such as tumor-associated receptors; unbound probe contributes to background signal, lowering the SNR. “Smart” probes (**C**) are activated by enzymatic conversion yielding a strong fluorescence increase once the fluorochromes are released from the backbone (*right*). Tumor-associated proteases can be detected using this approach (reprinted with permission from Bremer et al. 2003)

contrast agents are accumulated at the target over time offering a strong signal in the region of interest (Fig. 1). However, there is – especially in the early phase after injection – a fair amount of unbound circulating material contributing to background signal and therefore lowering the SNR. Different targeted contrast agents have been described and experimentally validated using large molecules such as antibodies (AB) or AB fragments to small peptide derivatives serving as tissue-specific affinity ligands (e.g., bombesin, somatostatin, or the folated receptor; (Becker et al. 2001; Licha et al. 2002)).

"Smart" optical probes change their signal characteristics upon interaction with the target. They ideally undergo a status of zero signal in their native state to a strong signal after target interaction. A class of smart optical contrast agents was recently described, which undergo conformational changes after cleavage by various enzymes (Weissleder et al. 1999; Fig. 1 C). In 1999, the first autoquenched fluorescent probe was developed, which is converted from a non-fluorescence to fluorescence state by proteolytic activation (Weissleder et al. 1999).

The first generation of this smart contrast agent consists of a long circulating carrier molecule (poly-lysine backbone, ~450 kDa) shielded by multiple methoxy-polyethylene-glycol sidechains (PLL-MPEG). Twelve to 14 cyanine dyes (Cy 5.5) are loaded onto this carrier molecule resulting in a signal quench due to fluorescence resonance energy transfer (FRET; Weissleder et al. 1999). Thus, in its native state the molecule exhibits very little to no fluorescence, whereas after enzymatic cleavage a strong fluorescence signal increase (up to several 100-fold) can be detected (dequenching; Fig. 1 C). Inhibition experiments revealed that this first generation of protease-sensing optical probes are activated mainly by lysosomal cysteine or serine proteases such as cathepsin-B (Weissleder et al. 1999).

The selectivity of this smart optical probe can be tailored to other enzymes by insertion of enzyme-specific peptide stalks between the carrier and the fluorochromes. In order to impart MMP-2 selectivity, for instance, a peptide stalk with the following sequence: -Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys- was inserted between the backbone and the fluorochrome. This peptide sequence is recognized by MMP-2 with a high affinity resulting in an efficient dequenching of the completely

assembled MMP-2 probe by the purified enzyme. A control-probe that was synthesized using a scrambled peptide sequence (-Gly-Val-Arg-Leu-Gly-Pro-Gly-Lys-) remained quenched after incubation with the enzyme. Besides MMPs, other enzyme systems such as cathepsin-D or thrombin could be targeted using this approach (Weissleder et al. 1999; Bremer et al. 2001 a, b).

9.3.1 Oncological Imaging

Different proteases are known to be key players in a whole variety of pathologies ranging from carcinogenesis to immune diseases (Edwards and Murphy 1998). It is known that various proteases such as cathepsins and matrix-metalloproteinases are involved in a cascade of enzymes, which finally yields to the digestion of the extracellular matrix and thus local as well as metastatic tumor cell infiltration (Edwards and Murphy 1998; Fang et al. 2000; Folkman 1999; Aparicio et al. 1999; Herszenyi et al. 1999; Koblinski et al. 2000). Indeed, clinical data suggest that tumoral protease burden correlates with clinical outcome (McCarthy et al. 1999; Kanayama et al. 1998; Davidson et al. 1999).

The first experimental studies using a LX-1 tumor xenograft model showed that even small tumor nodules could be sensitively contrasted with the cathepsin-B sensing optical probe, suggesting that protease imaging might be a feasible approach for early cancer detection (Weissleder et al. 1999; Mahmood et al. 1999). Indeed, there is evidence that protease overexpression is an early step involved in malignant cell transformation. In colon cancer, for example, cathepsin-B and matrix-metalloproteinases have been found to be upregulated within the invasive tumor margins (Campo et al. 1994; Emmert-Buck et al. 1994; Khan et al. 1998 a, b). In line with this observation is the fact that even premalignant gastrointestinal lesions could be detected by enyzme-sensing molecular beacons (Marten et al. 2002). Dysplastic colon polyps in aged $APC^{Min/+}$ mice serving as a model for spontaneous adenomatosis coli could be sensitively detected using the cathepsin-B sensing optical probe (Marten et al. 2002). Even lesions in the submillimeter range (50 µm in diameter), which were invisible with white-light inspection, could be visualized

using this approach. Lesion conspicuity could be significantly enhanced compared to visual inspection, yielding a sensitivity of about 96% and a specificity of about 93% (Marten et al. 2002).

Highly invasive cancers have been linked to a higher load of proteases in both clinical and experimental investigations (McCarthy et

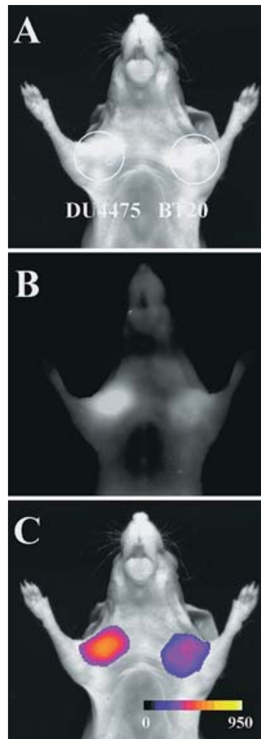

Fig. 2 A–C. Tumor differentiation with NIRF imaging using protease-sensing probes. Fluorescence reflectance imaging using a cathepsin-B sensing optical probe. The animal was implanted with a highly invasive (DU4475; *right chest*) and a well-differentiated breast adenocarcinoma (BT20; *left chest*); light image (**A**), raw NIRF image (**B**), and color-encoded NIRF signal (AU of NIRF-intensity) superimposed on light image (**C**). Due to a higher protease load, the invasive breast lesion activated the probe more efficiently, resulting in a higher NIRF signal of the tumor (**B**, **C** reprinted with permission from Bremer et al. 2002)

al. 1999; Kanayama et al. 1998; Davidson et al. 1999; Kuniyasu et al. 1999; Murray et al. 1996; Sakakibara et al. 1999). Therefore, noninvasive tumor grading might be another application of protease imaging. In a breast cancer xenograft model, a highly invasive human adenocarcinoma (DU4475) was tested against a well-differentiated breast adenocarcinoma (BT20; Bremer et al. 2002; Fig. 2). In accordance with the literature, the protease load (cathepsin-B) of the DU4475 tumor cells was about 1.7-fold higher compared to the well-differentiated adenocarcinoma. Fluorescence reflectance imaging (FRI) of the tumors revealed significantly stronger probe activation by the highly invasive compared to the well-differentiated tumor (Bremer et al. 2002). A grading of tumor types according to protease load can therefore be envisioned using protease sensing optical probes.

Treatment effects of novel protease inhibitors can also be visualized using protease-sensing optical probes (Bremer et al. 2001 a, b). Nude mice implanted with MMP overexpressing fibrosarcomas (HT1080) were treated with an MMP-protease inhibitor (AG3340, Aguron/Pfizer, San Diego, CA) and imaged by FRI using the MMP-sensing optical probe (see Sect. 9.3). Treatment resulted in a significant reduction of protease-induced NIRF signal which could be detected as early as 48 h after initiation of the treatment, long before phenotypical changes (such as regression of tumor size, rarefication of vasculature, etc.) can be seen (Bremer et al. 2001 b). Thus protease imaging is a very sensitive means to assess novel antitumor therapies (Bremer et al. 2001 b).

9.4 Nononcological Imaging

Protease imaging can potentially be applied to imaging the activity of autoimmune diseases such as rheumatoid arthritis (Ji et al. 2002). In a model of inducible rheumatoid arthritis, FRI with a cathepsin-B sensing probe detected inflammatory activity in the joints as early as 20 h after disease induction while clinical manifestations of the disease were still discrete (Ji et al. 2002).

Inflammatory activity in atherosclerotic soft plaques were recently visualized by FRI using the cathepsin B probe (Chen et al.

2002). Twenty four hours after probe injection, aortic atherosclerotic plaques showed strong probe activation in ApoE-deficient mouse strains that were kept on a Western-style, lipid-rich diet (Chen et al. 2002). There was a clear colocalization of tissue fluorescence and soft plaques in the mouse aorta. Cathepsin B expression was confirmed by immunohistochemistry of plaques sections. Moreover, thrombin activity was recently visualized using a dedicated thrombin-sensitive optical probe (Tung et al. 2002). Since thrombin is a key enzyme in hemostasis and thrombotic events, a screening for clotting disorders or thrombosis might be possible with this molecular sensor (Tung et al. 2002).

9.5 Potential Clinical Applications

With the advent of three-dimensional, quantitative imaging methods for detection of fluorochrome distribution such as fluorescence-mediated tomography (FMT; Ntziachristos and Weissleder 2001; Ntziachristos et al. 2001, 2002 a, b), a wide range of clinical applications of optical imaging can be envisioned (Fig. 3). Numerical simulations based on current laboratory data suggest that FMT can be upscaled for human use (Ntziachristos et al. 2002 c). Moreover, in a clinical study combing gadolinium-enhanced MRI with indocyanine-green-enhanced diffuse optical tomography (DOT), breast lesions could be optically resolved, which serves as a proof of principle for the applicability of this concept for human use.

Combining detailed anatomical resolution by MRI or CT with molecular information generated by optical signatures or contrast media, respectively, would greatly enhance the sensitivity and, most of all, the specificity of biomedical imaging. Contrast-enhanced optical breast imaging will be the first application to come and the first clinical studies are currently underway. However, other aspects of cancer imaging can potentially be addressed using FMT and smart or targeted optical probes including musculoskeletal, head and neck, and pediatric imaging applications.

Besides tomographic sensors, a combination of endoscopic or laparoscopic devices with optical fibers for fluorochrome detection should be feasible to build. Moreover, handheld scanning devices

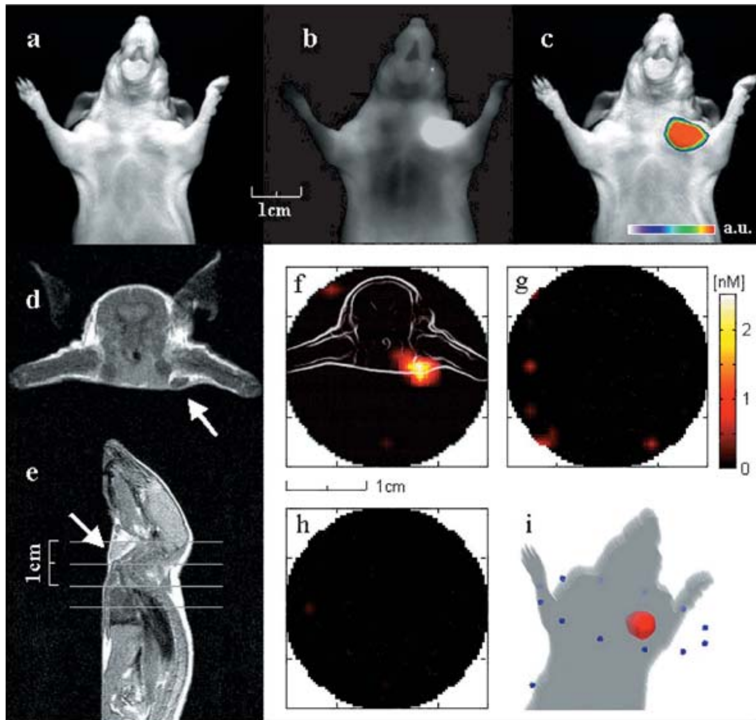

Fig. 3 a–i. Fluorescence mediated tomography (FMT). FMT allows for three-dimensional, quantitative imaging of fluorochrome distribution in vivo. **a** Intrinsic light image obtained with reflectance imaging. **b** Fluorescence image obtained with reflectance imaging. **c** Superposition of **b** onto **a** after threshold application. **d** MRI axial slice at the level of the uppermost FMT slice acquired, with an *arrow* denoting the tumor location. **e** MRI sagittal slice with lines denoting the three-slice volume segmentation assumed by FMT. A fibrosarcoma was implanted in the mammary fat pad of a nude mouse. An *arrow* denotes the tumor location. **f–h** Reconstructed FMT slices. The outline of the axial MRI slice is shown superimposed in **f** for registration purposes. **i** Volume rendering of the FMT dataset (*red*). *Blue spheres* denote the locations of the most superior ring of detectors. Note that three-dimensional, quantitative assessment of fluorochrome distribution is feasible using FMT (reprinted with permission from Ntziachristos et al. 2002 a)

comparable to ultrasound probes may be useful to screen for superficial fluorochrome detection.

In summary, optical imaging techniques provide excellent tools for probing molecular information in vivo both for experimental and clinical applications.

References

Aparicio T, Kermorgant S, Dessirier V, Lewin MJ, Lehy T (1999) Matrix metalloproteinase inhibition prevents colon cancer peritoneal carcinomatosis development and prolongs survival in rats. Carcinogenesis 20:1445–1451

Becker A, Hessenius C, Licha K, Ebert B, Sukowski U, Semmler W, Wiedenmann B, Grotzinger C (2001) Receptor-targeted optical imaging of tumors with near-infrared fluorescent ligands. Nat Biotechnol 19:327–331

Bremer C, Tung CH, Weissleder R (2001) In vivo molecular target assessment of matrix metalloproteinase inhibition. Nat Med 7:743–748

Bremer C, Bredow S, Mahmood U, Weissleder R, Tung CH (2001 a) Optical imaging of matrix metalloproteinase-2 activity in tumors: feasibility study in a mouse model. Radiology 221:523–529

Bremer C, Tung CH, Bogdanov A, Jr, Weissleder R (2002) Imaging of differential protease expression in breast cancers for detection of aggressive tumor phenotypes. Radiology 222:814–818

Bremer C, Ntziachristos V, Weissleder R (2003 b) Optical-based molecular imaging: contrast agents and potential medical applications. Eur Radiol 13:231–243

Campo E, Munoz J, Miquel R, Palacin A, Cardesa A, Sloane BF, Emmert-Buck MR (1994) Cathepsin B expression in colorectal carcinomas correlates with tumor progression and shortened patient survival. Am J Pathol 145:301–309

Chen J, Tung CH, Mahmood U, Ntziachristos V, Gyurko R, Fishman MC, Huang PL, Weissleder R (2002) In vivo imaging of proteolytic activity in atherosclerosis. Circulation 105:2766–2771

Davidson B, Goldberg I, Kopolovic J, Lerner-Geva L, Gotlieb WH, Ben-Baruch G, Reich R (1999) MMP-2 and TIMP-2 expression correlates with poor prognosis in cervical carcinoma – a clinicopathologic study using immunohistochemistry and mRNA in situ hybridization. Gynecol Oncol 73:372–382

Edwards DR, Murphy G (1998) Cancer. Proteases – invasion and more. Nature 394:527–528

Emmert-Buck MR, Roth MJ, Zhuang Z, Campo E, Rozhin J, Sloane BF, Liotta LA, Stetler-Stevenson WG (1994) Increased gelatinase A (MMP-

2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. Am J Pathol 145:1285–1290

Fang J, Shing Y, Wiederschain D, Yan L, Butterfield C, Jackson G, Harper J, Tamvakopoulos G, Moses MA (2000) Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proc Natl Acad Sci USA 97:3884–3889

Folkman J (1999) Angiogenic zip code. Nat Biotechnol 17:749

Herszenyi L, Plebani M, Carraro P, De Paoli M, Roveroni G, Cardin R, Tulassay Z, Naccarato R, Farinati F (1999) The role of cysteine and serine proteases in colorectal carcinoma. Cancer 86:1135–1142

Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, Takahashi K, Holers VM, Walport M, Gerard C, Ezekowitz A, Carroll MC, Brenner M, Weissleder R, Verbeek JS, Duchatelle V, Degott C, Benoist C, Mathis D (2002) Arthritis critically dependent on innate immune system players. Immunity 16:157–168

Kanayama H, Yokota K, Kurokawa Y, Murakami Y, Nishitani M, Kagawa S (1998) Prognostic values of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression in bladder cancer. Cancer 82:1359–1366

Khan A, Krishna M, Baker SP, Banner BF (1998 a) Cathepsin B and tumor-associated laminin expression in the progression of colorectal adenoma to carcinoma. Mod Pathol 11:704–708

Khan A, Krishna M, Baker SP, Malhothra R, Banner BF (1998 b) Cathepsin B expression and its correlation with tumor-associated laminin and tumor progression in gastric cancer. Arch Pathol Lab Med 122:172–177

Koblinski JE, Ahram M, Sloane BF (2000) Unraveling the role of proteases in cancer. Clin Chim Acta 291:113–135

Kuniyasu H, Ellis LM, Evans DB, Abbruzzese JL, Fenoglio CJ, Bucana CD, Cleary KR, Tahara E, Fidler IJ (1999) Relative expression of E-cadherin and type IV collagenase genes predicts disease outcome in patients with resectable pancreatic carcinoma. Clin Cancer Res 5:25–33

Licha K, Riefke B, Ebert B, Grotzinger C (2002) Cyanine dyes as contrast agents in biomedical optical imaging. Acad Radiol 9 [Suppl 2]:S320–322

Mahmood U, Tung CH, Bogdanov A, Jr, Weissleder R (1999) Near-infrared optical imaging of protease activity for tumor detection. Radiology 213:866–870

Marten K, Bremer C, Khazaie K, Sameni M, Sloane B, Tung CH, Weissleder R (2002) Detection of dysplastic intestinal adenomas using enzyme-sensing molecular beacons in mice. Gastroenterol 122:406–414

McCarthy K, Maguire T, McGreal G, McDermott E, O'Higgins N, Duffy MJ (1999) High levels of tissue inhibitor of metalloproteinase-1 predict poor outcome in patients with breast cancer. Int J Cancer 84:44–48

Murray GI, Duncan ME, O'Neil P, Melvin WT, Fothergill JE (1996) Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. Nat Med 2:461–462

Ntziachristos V, Weissleder R (2001) Experimental three-dimensional fluorescence reconstruction of diffuse media by use of a normalized Born approximation. Optics Lett 26:893–895

Ntziachristos V, Tung C, Bremer C, Weissleder R (2001) Fluorescence-mediated tomography resolves protease activity in vivo. Nat Med 8:757–760

Ntziachristos V, Bremer C, Weissleder R (2002 a) Fluorescence imaging with near-infrared light: new technological advances that enable in vivo molecular imaging. Eur Radiol 13:195–208

Ntziachristos V, Bremer C, Graves EE, Ripoll J, Weissleder R (2002 b) In vivo tomographic imaging of near-infrared fluorescent probes. Mol Imaging 1:82–88

Ntziachristos V, Ripoll J, Weissleder R (2002 c) Would near-infrared fluorescence signals propagate through large human organs for clinical studies. Optics Lett 27:527–529

Sakakibara M, Koizumi S, Saikawa Y, Wada H, Ichihara T, Sato H, Horita S, Mugishima H, Kaneko Y, Koike K (1999) Membrane-type matrix metalloproteinase-1 expression and activation of gelatinase A as prognostic markers in advanced pediatric neuroblastoma. Cancer 85:231–239

Tung CH, Gerszten RE, Jaffer FA, Weissleder R (2002) A novel near-infrared fluorescence sensor for detection of thrombin activation in blood. Chembiochem 3:207–211

Weissleder R (2001) A clearer vision for in vivo imaging. Nat Biotechnol 19:316–317

Weissleder R, Tung CH, Mahmood U, Bogdanov A, Jr (1999) In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. Nat Biotechnol 17:375–378