Advances in Brief

Immunoscintigraphic Detection of the ED-B Domain of Fibronectin, a Marker of Angiogenesis, in Patients with Cancer

Monica Santinaria, Giovanni Moscatelli, Giuseppe L. Viale, Leonardo Giovannoni, Giovanni Neri, Francesca Viti, Alessandra Leprini, Laura Borsi, Patrizia Castellani, Luciano Zardi, Dario Neri, and Pietro Riva

Servizio di Medicina Nucleare, Ospedale M. Bufalini, 47023 Cesena, Italy [M. S., G. M., P. R.]; Division of Neurosurgery Di.S.C.A.T. Department of Surgery, University of Genoa, Medical School, 16132 Genoa, Italy [G. L. V.]; Philegen S.r.l., 53100 Siena, Italy [L. G., G. N., F. V., A. L.]; Istituto Nazionale per la Ricerca sul Cancro, 16132 Genova, Italy [L. B., P. C., L. Z.;]; Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology Zurich, CH-8057 Zurich, Switzerland [D. N.]; and Istituto Oncologico Romagnolo, Forli, Italy [P. R.]

Abstract

Purpose: ED-B fibronectin is expressed only during angiogenic processes and in tissues undergoing growth and/or extensive remodeling. We demonstrated previously the possibility to target and selectively deliver therapeutic substances to tumor vasculature in experimental animal models using a human recombinant antibody fragment, L19, specific for the ED-B domain of fibronectin. Here we evaluate the possibility of targeting primary tumors and metastatic lesions in cancer patients through immunoscintigraphy using \(^{123}\)I-labeled dimeric L19 [L19(scFv)\(_2\)].

Experimental Design: Twenty patients (34–79 years of age) with lung, colorectal, or brain cancer, whose tumors had been confirmed by imaging techniques and/or histologically, were admitted to the immunoscintigraphic investigation.

Results: The dimeric L19 antibody selectively localized in tumor lesions in aggressive types of lung cancer and colorectal cancer. Because ED-B fibronectin is expressed only during angiogenic processes and in tissues undergoing growth and/or extensive remodeling, L19(scFv)\(_2\) is able to distinguish between quiescent and actively growing lesions. No side effects were observed.

Conclusions: The ability of L19(scFv)\(_2\) to target tumors in patients provides the foundations for new therapeutic applications, in which the L19 antibody is engineered to selectively deliver bioactive molecules to primary tumors as well as to metastases.

Introduction

Angiogenesis, i.e., the proliferation of new blood vessels from pre-existing ones, is a characteristic feature of aggressive solid tumors and other relevant disorders, such as age-related macular degeneration, diabetic retinopathy, and rheumatoid arthritis (1–3). The switch of solid tumors from a poorly vascularized state to a condition in which exuberant angiogenesis provides tumor cells with oxygen and nutrients often corresponds to the onset of a more aggressive phenotype (4).

The noninvasive imaging of angiogenesis and tissue remodeling processes in vivo would prove to be dramatically advantageous over current methods, because it would yield information about both the location and the growth dynamics of lesions. Because neovasculature and tissue remodeling are required for the growth of all aggressive solid tumors, the same imaging approach could be used for different types of cancer. Furthermore, the proven ability of a molecule (e.g., a human monoclonal antibody fragment) to selectively localize in new blood vessels or actively remodeling tissues would herald a number of therapeutic strategies in which bioactive compounds are selectively targeted to angiogenic sites (5, 6).

Imaging of angiogenesis in animal models and in patients with cancer has been attempted, using computed tomography, MRI, ultrasound, and scintigraphic techniques, to assess changes in vascular permeability and tumor blood flow during antiangiogenic therapy (7). However, a quantitative distinction between vascularity and angiogenesis would be expedient, because a number of benign tumors are known to be highly vascular despite a low rate of new blood vessel formation (8). Imaging of angiogenesis using molecular targeting agents (e.g., antibody fragments) may provide the means to achieve this goal.

Specific ligands of the integrin \(\alpha\)v\(\beta\)3 have been used for imaging of angiogenesis in preclinical models (9, 10), but results from an immunoscintigraphic clinical trial with the humanized anti\(\alpha\)v\(\beta\)3 antibody Vitaxin has thus far been disappointing (11). We focused our attention on the ED-B\(^3\) of fibronectin, a marker of angiogenesis and tissue remodeling (8, 12–15). This sequence of 91 amino acids, identical in mouse, rat, rabbit, dog, and humans, can be inserted into the fibronectin molecule by a mechanism of alternative splicing at the level of the primary fibronectin transcript. Fibronectin containing ED-B (B-FN) ac-
cumulates around neovascular structures in aggressive tumors and other tissues undergoing angiogenesis and remodeling, such as neoplasia, some ocular structures in pathological conditions, and fetal tissues, but is otherwise undetectable in normal adult tissues with the exception of the female reproductive system, where tissue remodeling and angiogenesis are recurrent physiological processes.

To date, the production of monoclonal antibodies directly recognizing the ED-B domain in B-FN has not been possible using hybridoma technology because of tolerance. We have overcome this problem, using large synthetic antibody repertoires (16–19), in combination with phage display (20, 21) or iterative colony filter screening (22). Antibodies specific to B-FN selectively target the neovascularulature in vivo, as shown in tumor-bearing mice (23–26) and in rabbit models of ocular angiogenesis (27), thus underpinning the possibility to selectively deliver therapeutic molecules to new blood vessels. The scFv(L19) antibody fragment, with picomolar affinity for the ED-B domain (21), was chemically coupled to a photosensitizer, and was shown to mediate the selective and complete occlusion of ocular neovascularulature in a rabbit model after irradiation with near infrared light (27). Nilsson et al. (28) reported that the fusion protein composed of L19 and the extracellular domain of tissue factor mediates the selective thrombosis of new blood vessels in different types of murine tumor models; scFv(L19) has been shown recently to dramatically increase the therapeutic index of cytokines when used to deliver these to the tumor neovascularulature (29, 30).

In this article we report on the immunoscintigraphic findings obtained using the noncovalent homodimeric form of L19 scFv [L19(scFv)2] labeled with the γ-emitter 123I in 20 patients with cancer.

Patients and Methods

Antibody Preparation, Immunohistochemistry, and Antibody Radioiodination. The scFv(L19) antibody fragment was produced in the supernatant of Escherichia coli as described (24), according to the guidelines for preparation of recombinant DNA products in Phase I trials in patients with Cancer of the Cancer Research Campaign (31–33). In short, the antibody was affinity purified from the bacterial supernatanton a ED-B-Agarose resin (24), followed by dialysis in 10 mM HEPES (pH 7.0) at 1 mg/ml concentration; the resulting protein solution mainly contained the noncovalent homodimer of the scFv that was separated from the monomer by cation exchange chromatography on a Resource S column (Amersham Pharmacia, Uppsala, Sweden). The L19(scFv)2 gave a single band in SDS-PAGE loading up to 20 μg/ lane, and was a pure noncovalent homodimer, as judged by size-exclusion chromatography. Purity, immunoreactivity, identity, and absence of endotoxins were additionally tested by chromatography, matrix-assisted desorption ionization-time of flight, ELISA, BIACore, and Limulus amoeboocyte lystate, as well as in tumor-targeting experiments in tumor-bearing mice and in a rabbit-based pyrogen test. Absence of acute toxicity at doses >10-fold higher than the ones used in patients was assessed in mice, rats, and guinea pigs, under good laboratory practice conditions. The final purified antibody bulk was pooled, sterile filtered, and frozen in aliquots in GMP conditions by Bioreliance (Stirling, Scotland) at A280 = 1.14 in saline solutions.

Immunohistochemical studies were performed as described (8, 20), using 5-μm cryostat sections of freshly frozen tumor specimens, including samples from 2 patients with newly diagnosed lesions, who had been imaged previously with 123I-L19(scFv)2.

Immediately before the scintigraphic studies, the L19(scFv)2 antibody was labeled with 123I (Sorin Amersham, Saluggia, Italy), using the chloramine-T method (31). The radiodinated antibody was purified from the reaction mixture by gel filtration (PD-10 columns equilibrated with saline solution; Amersham Pharmacia Biotech) and 0.22-μm sterile filtered.

The 123I incorporation ranged between 70% and 90%, as judged by radioactive counting of the PD-10 eluate, thin layer chromatography, and high-performance liquid chromatography gel-filtration. In all of the cases tested, the immunoreactivity was >85%, as measured by affinity chromatography on an ED-B resin, as described (27), with a single exception where it was ~50%.

Immunoscintigraphic Procedures. Twenty patients (34–79 years of age) with brain, lung, or colorectal cancer [2 brain tumors: 1 pycnotic astrocytoma, 1 glioblastoma multiforme; 16 lung cancers: 7 squamous cell carcinomas, 4 small cell carcinomas (2 of which with liver metastases), 1 large cell anaplastic carcinoma, 3 adenocarcinomas (1 of which bronchioloalveolar), 1 sarcoma; and 2 colorectal carcinomas with liver metastases], whose tumor had been confirmed by imaging techniques and/or histologically, were admitted to the immunoscintigraphic investigation after giving their informed consent. One mg of 123I-L19(scFv)2 (5–14 mCi) in 10 ml of 0.9% sodium chloride was administered i.v. over 2 min, followed by flushing with 0.9% sodium chloride. The planar images were obtained 4 and 24 h after the i.v. infusion of the radiolabeled monoclonal antibody. A computer-assisted, large field of view GE gamma camera, equipped with a low energy and high-resolution collimator, was used. Brain, chest, abdomen, and pelvis, in anterior and posterior view, were imaged by collecting 300 k counts. SPECT examination was carried out by using the same gamma camera. If appropriate, images at other time points were collected. In hospitalized patients the thyroid uptake of possible free 123I was prevented by administration of potassium perchlorate (Pertiroid; PIAM, Genoa, Italy), 400 mg three times a day starting on day −4 and continuing till day +1. The study was performed according to good clinical practice standards, in compliance with the Italian Decreto Legislativo 26 May 2000, n. 187, regulating clinical investigations, which follows the European Community guideline 97/43/EURATOM. Immunoscintigraphic studies with scFv(L19) received authorization number: 800/II/L.27.15/1172 of the Italian Ministry of Health. The dose to the target tumor and to major organs was calculated according to the methodology described previously (34).

Results

Expression of B-FN in Different Tumor Types. Extensive immunohistochemical analyses of B-FN expression in tumors have been described elsewhere, and practically all of the solid tumors show the presence of ED-B (8, 13–15, 35, 36). The
tumor types that, based on immunohistochemical findings, normally show a particularly abundant presence of ED-B are lung cancers, high-grade astrocytomas (37), and liver metastases. By contrast, ED-B was constantly undetectable in normal lung, brain, and liver specimens.

Fig. 1 shows the immunohistochemical findings for some of the tumor types that were investigated in this study. A striking contrast in ED-B staining can be observed between aggressive brain tumors (e.g., glioblastoma; Fig. 1a), where practically all of the blood vessels reacted positively with the anti-ED-B antibody, and low-grade astrocytomas (e.g., pilocytic astrocytoma; Fig. 1b) where ED-B was undetectable, regardless of the vascular density (8). Fig. 1d shows a section from a specimen of a lung squamous carcinoma stained using the L19(scFv)2; a strong and diffuse staining of the tumor stroma is clearly visible.

Fig. 1e shows a section of a large cell anaplastic lung carcinoma stained with the L19(scFv)2; it is easier to observe ED-B-positive vascular structures here, because the connective components of the tumor stroma in this tumor type are less abundant compared with squamous lung cancer. By contrast, no ED-B staining could be detected in normal lung specimens (Fig. 1f).

We also investigated liver metastases of colorectal cancer, and found strong positive staining using the recombinant antibody to ED-B (Fig. 1c). These immunohistochemical findings prompted us to initiate our immunoscintigraphic trial in patients with lung cancer, cerebral glioma, or liver metastasis.

**Antibody Radiolabeling and Fractional Blood Clearance.** L19(scFv)2 was radiolabeled with 123I and given i.v. to 20 patients with brain, lung, or colorectal cancer. The injected dose of 123I-labeled L19(scFv)2 ranged from 5 to 14 mCi.
(185–518 MBq) corresponding to 1–1.5 mg of protein with a single exception where the injected dose was 0.4 mg. A biphasic clearance profile of radioiodinated L19(scFv)2 from blood was observed. Fitting of the curve with a biexponential function yielded half-lives of 35 min (α phase, accounting for 86% of the injected dose) and 5.2 h (β phase, accounting for 14% of injected dose). Antibody clearance was mediated principally by the kidneys, as determined by counting of urine samples. Begent et al. (31) reported previously that the clearance of a radiolabeled scFv anticarcinoembryonic antigen injected in patients was kidney mediated. However, Begent et al. (31) used a monomeric scFv (27 kDa), whereas here we used the homodimeric L19(scFv)2 of ~57 kDa. Size exclusion chromatography analysis of blood samples at different time points showed that >80% of radioactivity in blood was associated with radiolabeled L19(scFv)2 (85% at 3.5 h; 84% at 6.5 h; and 82% at 22 h), and immunoreactivity was ~50% at 3 h after injection.

**Immunoscintigraphic Study.** A total of 20 cancer patients were injected with 123I-radiolabeled L19(scFv)2. All of the patients tolerated the scFv injection well without showing side effects. No early or late allergic reaction was observed. The hematological parameters were not affected, and no adverse effects according to common toxicity criteria were seen (38). Sixteen of 20 patients showed different levels of antibody accumulation either in the primary tumors or metastases. The four completely negative scans were from patients with a lung sarcoma, a bronchioalveolar carcinoma, a squamous cell lung carcinoma, and a low-grade pylocytic astrocytoma. The negative results of the low-grade astrocytoma were expected, because this type of tumor does not express ED-B. We have no explanation at the moment for the 3 other negative patients, other than the possibility that the tumors were in a quiescent phase.

Fig. 2 shows representative anterior planar scans, at 18 h, of the thorax of patients injected with 123I-labeled L19(scFv)2. Fig. 2a shows the scan of a patient with liver metastases of colorectal cancer that typically exhibited a strong and selective antibody uptake in the liver lesions. Fig. 2b shows the scan of the thorax of a small cell lung carcinoma patient with a miliary involvement of both lungs, revealing a diffuse accumulation of the radioiodinated L19(scFv)2 in both lungs.

**Brain Tumors.** The SPECT analysis of Fig. 3a shows a strong and selective accumulation of 123I-labeled L19(scFv)2 in the tumor mass of a patient with recurrent glioblastoma, growing within the postoperative cavity and in the adjacent tissue (Fig. 3b). By contrast, Fig. 3c shows no selective accumulation in the brain of a patient with a benign brain tumor (pylocytic astrocytoma), which could be removed only partially by brain surgery (Fig. 3d). The differences in antibody uptake correlated with the different levels of antigen expression always found in these pathologies, as exemplified in Fig. 1, a and b, and with the differences in the integrity of the blood-brain barrier in these tumor types (39).

**Lung Cancer.** Fig. 4 shows SPECT images obtained 6 h after injection of radioiodinated L19(scFv)2 in a patient with a newly diagnosed large cell anaplastic lung carcinoma who had not received chemotherapy previously. A selective antibody accumulation in the tumor lesion was clearly detectable. The tumor size was estimated to be 5 × 7 × 5.5 cm (on the basis of SPECT images), and was consistent with the tumor diameter estimate obtained by CT. Immunohistochemistry of the tumor, after surgical removal, confirmed a strong and diffuse ED-B expression in the tumor stroma, which is rich in vascular structures (Fig. 1e).

**Liver Metastases.** Fig. 5 shows an array of SPECT scans from patients who had undergone surgery for removal of the primary tumor and who later presented with bulky liver metastases. Fig. 5a shows a selective antibody accumulation in a large (8 cm) liver metastasis of a colon carcinoma 21 h later.
after injection. The lesion was clearly detectable already 6 h after injection, with tumor:background ratios increasing at 21 h. Tumor:normal tissue and tumor:liver ratios were 3.7 and 1.6 at 6 h, and 9.2 and 4.6 at 21 h, respectively. At this time point, the antibody dose delivered to the tumor was greater than for all of the other organs, including kidneys. Fig. 5b shows the immunoscintigraphic detection of a liver metastasis from a small cell lung carcinoma, obtained 6 h after injection. Fig. 5c and d, show SPECT images of large liver metastases in the same patient, recorded 6 h after injection. A strong and selective antibody accumulation is visible in the peripheral part of the lesion but not in the necrotic center of the tumor mass, which yielded a characteristic doughnut-shape staining pattern. At this time point, tumor:normal tissue (soft tissue of the shoulder) and tumor: nontumoral part of the liver ratios were 4.8 and 1.9, respectively, calculated as reported previously (34).

Discussion
The tumor targeting performance of L19(scFv)2, a human antibody fragment with identical affinity for the ED-B domain of fibronectin from different animal species (21), was assessed previously by quantitative biodistribution analysis in a number of murine tumor models (24–26). The results presented in this article show the ability of L19(scFv)2, with its low molecular weight and its rapid renal clearance, to efficiently localize in aggressive primary tumors as well as metastases in patients with cancer. Accumulation of L19(scFv)2 in tumor lesions contrasts with the pharmacokinetic behavior of most chemotherapeutic drugs, which typically exhibit tumor:normal organ ratios as low as 1:10–1:20 at different time points after i.v. injection (40). Moreover, L19(scFv)2 demonstrated an impressive ability to deliver bioactive agents (procoagulant factors, cytokines, cytotoxic agents, radionuclides, and photosensitizers) to tumors in syngeneic animal models, with dramatic therapeutic results (27–30).

This study, carried out on 20 patients, the majority of whom had colorectal or lung cancer, clearly demonstrates that the antibody L19(scFv)2 selectively localized in patient tumors. This observation offers a number of important diagnostic and therapeutic prospects. Because the antigen recognized by the L19 recombinant antibody is always associated with angiogenesis and tissue remodeling, it is possible to obtain information on the growth potential of the lesion with a noninvasive proce-
dure, and relatively simple and commonly used tools. At present, this same kind of information is available only with sophisticated and expensive means such as positron emission tomography. Thus, immunoscintigraphy using L19(scFv)2 can provide important diagnostic information in the follow-up of low-grade (ED-B-negative) astrocytomas, which may switch to anaplastic astrocytomas that express large amounts of ED-B. The ability of 123I-labeled L19(scFv)2 to image high-grade, but not low-grade, astrocytomas indicates an avenue for the noninvasive discrimination between these two classes of brain tumors. Furthermore, immunoscintigraphy with L19(scFv)2 may prove useful in differentiating between postsurgery reactions, such as fibrosis, and tumor recurrence in a number of cancers, including lung cancer, where differential diagnosis is often impossible using classic radiodiagnostic procedures. We observed through immunohistochemistry that lymph nodes infiltrated by neoplastic cells show the presence of ED-B, whereas the noninfiltrated lymph nodes do not. Therefore, immunoscintigraphy using radiolabeled L19(scFv)2 would allow the noninvasive distinction between the two. Furthermore, the use of L19(scFv)2 could integrate approaches used for the identification of “sentinel lymph nodes” in breast cancer patients.

A particularly high accumulation of radiolabeled L19(scFv)2 was observed in hepatic metastatic lesions. If similar results were to be found in hepatocarcinomas, this otherwise untreatable cancer might well benefit from diagnostic and/or therapeutic approaches based on the use of radiolabeled L19(scFv)2. At the moment we have no evidence that immunoscintigraphy using L19(scFv)2 can identify micrometastases that are undetectable with current standard radiological procedures: the smallest lesion detected thus far is a subcentimetric liver metastasis (4–6 mm). However, the results of this study seem to suggest that L19(scFv)2 would be able to detect the more aggressive micrometastases, regardless of their size.

In view of the large number of ongoing clinical trials investigating antiangiogenic substances and in view of the antiangiogenic activity of most cytotoxic anticancer drugs (41, 42), the repeated imaging with 123I-labeled L19(scFv)2 might allow the better follow-up of patient response to treatment. The introduction of novel gamma cameras has lead to noteworthy improvements in immunoscintigraphic detection as well as the use of different radioisotopes (43–45). The use of positron emission tomography with suitable nuclides may enhance resolution and sensitivity, allowing a better three-dimensional localization of the tumor and more reliable quantitations. Furthermore, because ED-B is a pan-tumoral marker, new clinical trials on selected tumor types are necessary and are about to be started. The clinical studies presented in this article provide a strong rationale and a convincing incentive to rapidly introduce L19(scFv)2-based therapeutic fusion proteins into cancer clinical trials.

---

Acknowledgments

We thank Thomas Wiley for manuscript revision.

References


Immunoscintigraphic Detection of the ED-B Domain of Fibronectin, a Marker of Angiogenesis, in Patients with Cancer

Monica Santimaria, Giovanni Moscatelli, Giuseppe L. Viale, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/2/571

Cited articles
This article cites 43 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/2/571.full#ref-list-1

Citing articles
This article has been cited by 40 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/9/2/571.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/9/2/571.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.