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Quantitative estimates of angiogenic and anti-angiogenic activity by laser Doppler flowmetry (LDF) and near infra-red spectroscopy (NIRS)☆

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Abstract

The use of laser Doppler flowmetry (LDF) and near-infra-red spectroscopy (NIRS) for non-invasive *in vivo* measurements of angiogenic and anti-angiogenic activity in nude mice was evaluated. Angiogenic foci were induced in the skin by implantation of slow release pellets containing 200 ng basic fibroblast growth factor (bFGF). LDF and NIRS recordings from induced foci were significantly higher than placebo implants ($P<0.05$) and controls ($P<0.001$), proving that LDF and NIRS provide measures of angiogenic activity. Correspondingly, by these methods, an anti-angiogenic activity was significantly demonstrated in bFGF-stimulated animals treated with either the specific anti-angiogenic compound TNP-470 ($P<0.05$) or the anti-inflammatory agent dexamethasone ($P<0.001$). We conclude that LDF and NIRS, alone or in combination, are useful non-invasive tools for early evaluation of angiogenic and anti-angiogenic activity *in vivo*.

Keywords: Angiogenesis; LDF; NIRS; bFGF; TNP-470; Dexamethasone; Nude mice

1. Introduction

In vivo angiogenesis assays detect angiogenic and anti-angiogenic activity in living organisms, as opposed to *in vitro* assays based on, e.g. endothelial cell cultures. A prerequisite to effective *in vivo* estimates of angiogenesis and its modification is the availability of a quantitative, inexpensive, and routine angiogenesis assay. Ideally, with such assays, (1) it should be possible to distinguish between pre-existing host vessels and induced angiogenesis, (2) tissue damage should be avoided, since it may lead to secondary angiogenesis, and (3) the assay should permit long-term monitoring and preferably be non-invasive. The existing angiogenesis assays include microcirculatory window preparations, vascularisation into biocompatible implants, and excision of vascularised tissue. None of the existing angiogenesis assays fulfill all the abovementioned requirements, as recently reviewed by Jain and colleagues [1].

Laser Doppler flowmetry (LDF) provides estimates of local blood perfusion in superficial tissues for, e.g. skin [2]. LDF has a high time resolution and a measuring depth of approximately 2 mm [3]. The principle of this technique is based on the change in wavelength (Doppler shift) of the laser light that is reflected from moving subjects, such as erythrocytes, whereas the wavelength of light reflected from stationary subjects remain unchanged [4]. The Doppler-shifted light is converted into an arbitrary perfusion signal, which is approximately proportional to the mean blood cell velocity multiplied by the concentration of moving blood cells within the sampling volume. Comparison of LDF with the ^{133}xenon-clearance method has shown high correlation [5–7].

Near-infra-red spectroscopy (NIRS) can provide non-invasive and quantitative measurements of haemoglobin concentration and oxygenation. At 800 nm (the isoectic point of haemoglobin) the light absorption of a tissue is proportional to the haemoglobin concentration, disturbed only marginally by other chromophores of the tissue [8,9]. While the use of NIRS for measurements of total tissue haemoglobin concentration and haemoglobin

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oxygenation is well described, there is no prior description of the use of NIRS for quantification of angiogenesis.

Since angiogenesis is the formation of new capillaries, we hypothesised that measurements of the local blood perfusion and haemoglobin concentration over time in a specific area could provide a quantitative measure of the local angiogenesis. The aim of the present study was to utilise LDF and NIRS for non-invasive measurements of basic fibroblast growth factor (bFGF)-induced angiogenesis in the nude mouse, and to evaluate these principles as simple and objective methods for non-invasive *in vivo* evaluation of the effect of modifiers of angiogenesis.

2. Materials and methods

2.1. Animals

In total, 181 male 8-week athymic nude mice (NMRI-nu/nu) obtained from M&B (Ry, Denmark) were used. The mice were kept in groups of five in laminar air-flow benches and were fed sterile food pellets and water *ad libitum*. This body weight was regularly recorded. Institutional guidelines for animal welfare and experimental conduct were followed.

2.2. Induction of angiogenesis

Angiogenic foci were induced at defined regions of the skin by subcutaneous (s.c.) implantation of 3 mm in diameter slow-release pellets (Innovative Res., FL, USA) containing 200 ng of the angiogenic peptide bFGF [10] released at a constant rate over 10 days. The right hind leg was used due to good fixation of the pellet and lack of interference by respiratory movements (Fig. 1a). The unstimulated contralateral left hind leg was used to document that the effect of drugs on bFGF-induced angiogenesis was not a systemic effect. Prior to implantation, the animals were anaesthetised with ketamine/xylazine (10/100 mg/kg body weight (BW) s.c.). An incision of 1 cm was made through the skin approximately 2 cm from the hind leg and the pellet was tunnelled to its destination. Animals with unstable fixation of the implants on day 6 were excluded.

2.3. Inhibition of angiogenesis

A stock solution of 70% (v/v) ethanol and TNP-470 (Leo, Ballerup, Denmark), 10 mg/ml, was prepared and stored at 4°C. Immediately prior to treatment, the stock solution was diluted with 0.9% NaCl to obtain a dosing concentration of 1 mg/ml. TNP-470, 7 mg/kg every day (q.d.), was administered s.c. in the neck region starting immediately after bFGF implantation. TNP-470 is a potent inhibitor of angiogenesis, which has been shown to inhibit endothelial cell proliferation and migration, as well as tumour growth in several *in vivo* assays [11–13].

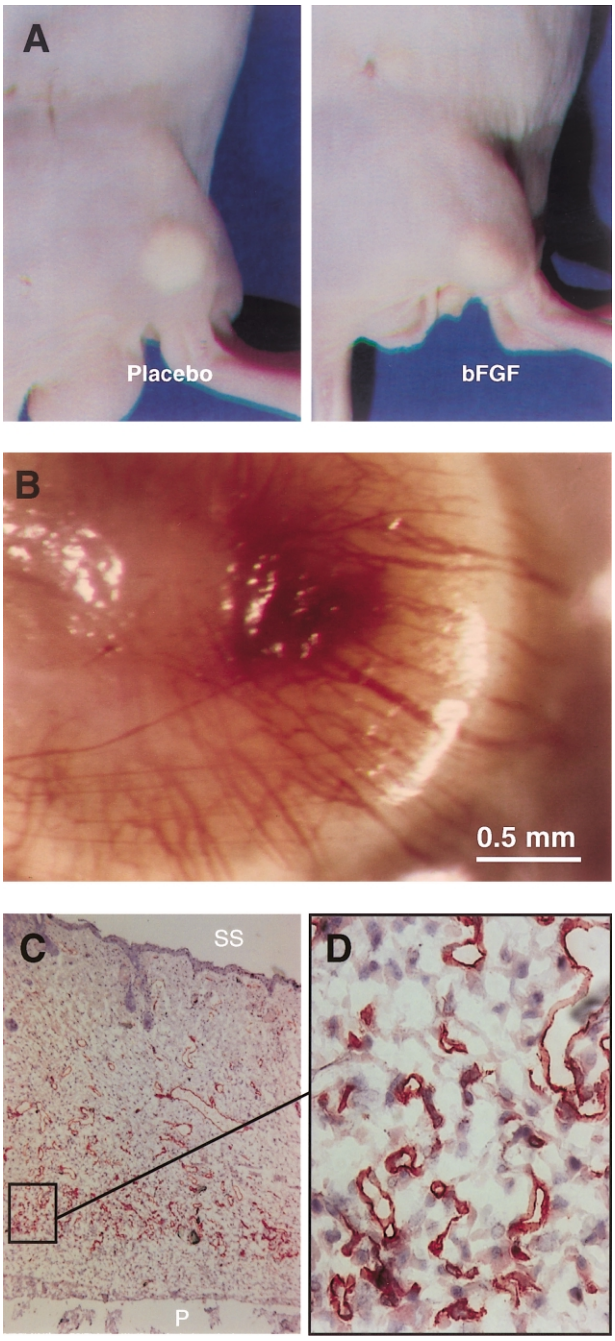

Fig. 1. Angiogenesis induced by basic fibroblast growth factor (bFGF). (A) Gross appearance of active (bFGF-releasing) and placebo pellet on day 7 after implantation; (B) low magnification photomicrograph (magnification×10) of the induced capillary network around a 3 mm in diameter slow release bFGF pellet; (C) representative cluster of differentiation (CD)31 immunostained cryosection (clone 390, Serotec Ltd. and MEC 13.3, Pharmingen) of a bFGF implant on day 7 after implantation showing the skin surface (SS) and the pellet (P) surrounded by abundant immature vascular structures (magnification ×50); (D) higher magnification photomicrograph (×400) of the induced capillary network around a bFGF pellet.

Dexamethasone (Decadron®), 10 mg/kg q.d., was given as intraperitonal (i.p.) injections starting the day before bFGF implantation. Dexamethasone is a potent anti-inflammatory agent with predominantly glucocorticoid action, which has been shown to inhibit sponge-induced angiogenesis in rats [14], as well as tumour angiogenesis [15].

2.4. LDF and NIRS recordings

The mice were anaesthetised 15 min before LDF and NIRS recordings to eliminate artifacts due to body movement. The experiments were performed during a constant room temperature of 25±1°C. During the measurements the mice were placed on a 37°C warm blanket. A micromanipulator was used to maintain a reproducible localisation of the LDF and NIRS probes perpendicular to the skin surface in close contact to the skin, yet carefully avoiding tissue compression. LDF recordings were performed on day 6, whereas NIRS recordings were performed on day 7 after implantation.

2.4.1. LDF

Laser light with the wavelength of 780 nm was transmitted to the skin by a 41°C heated custom-built probe with four integrated laser/receiver units (6 mm o.d., 250 μm fibre separation, time constant 0.2 s, PF415-175, Perimed, Sweden). The LDF probe was calibrated in motility standard solution before each experiment. The LDF signal was recorded continuously for three minutes and the perfusion unit (PU) was determined as the mean PU-value of the stabilised plateau, which was reached after approximately one minute.

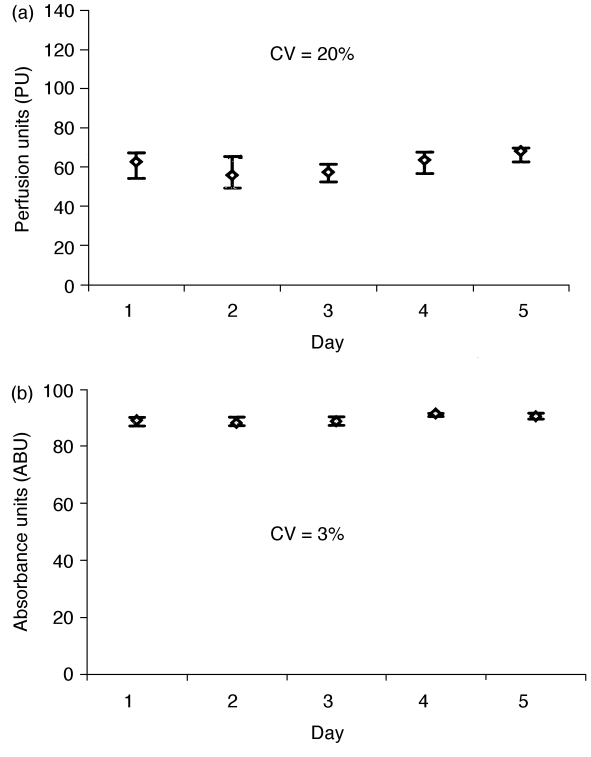

Fig. 2. Day-to-day variation of laser Doppler flowmetry (LDF) and near-infra-red spectroscopy (NIRS) recordings: (a) daily variation of LDF recordings on the left and right hind legs in nude mice ($n=10$); (b) daily variation of NIRS recordings on the left and right hind legs in nude mice ($n=10$). Median values and interquartile range. CV, coefficient of variation.

2.4.2. NIRS

The instrument was custom-built (Nuclear Magnetic Resonance (NMR)-Center, University of Copenhagen, Denmark) including a xenon flash as the light source (l4633 Hammamatsu, Near Infrared Spectrometer) and a photo diode (Siemens BPW21 photo diode) as the light detector. The coupling between the instrument and the animal was accomplished by a branched light guide. The diameter of the fused end was 3.0 mm. Interference filters were used to separate the 800±10 nm light. The NIRS instrument was calibrated before each experiment. Full absorption, i.e. zero signal was set to 100 absorbance units (ABU) and white paper (80 g/mm^2) was calibrated to 50 ABU. The signal was averaged over 30 seconds. The NIRS value was calculated as the median of five recordings.

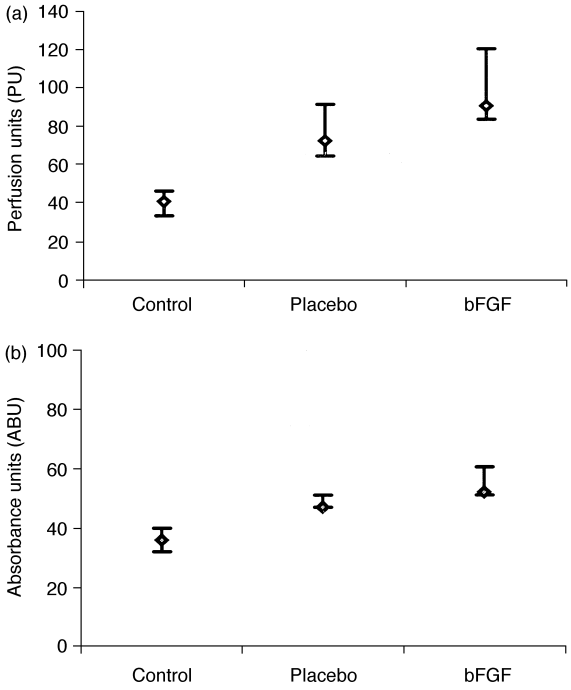

Fig. 3. Angiogenic activity assessed by LDF and NIRS: (a) LDF recordings on controls ($n=16$), i.e. on placebo pellets immediately after implantation, and on placebo ($n=23$) and bFGF ($n=23$) on day 6 after implantation. LDF recordings on bFGF were significantly higher than placebo ($P<0.05$) and controls ($P<0.001$), and placebo was significantly higher than controls ($P<0.001$); (b) NIRS recordings on controls ($n=16$), and on placebo ($n=15$) and bFGF ($n=15$) on day 7 after implantation. NIRS recordings on bFGF were significantly higher than placebo ($P<0.05$) and controls ($P<0.001$), and placebo was significantly higher than controls ($P<0.001$). Median values and interquartile range.

2.5. Statistical analysis

Data did not consistently show a Gaussian distribution. Therefore, statistical evaluations of differences in LDF and NIRS recordings between groups of animals were performed by a two-tailed Mann–Whitney U test. A P value of <0.05 was considered significant.

The coefficient of variation (CV) was used to compare the variability of the LDF- and NIRS techniques to the variability obtained in other studies. The CV was determined as the ratio of the standard deviation to the mean.

3. Results

Subcutaneous implantation of bFGF pellets induced angiogenesis. The new blood vessels grew around and above the implants. CD31 immunostained cryosections showed abundant angiogenic activity in the tissue surrounding the bFGF pellet (Fig. 1). Separate LDF and NIRS recordings on left and right hind legs showed no specific variation from day to day during a 5-day period (Fig. 2).

In bFGF-stimulated animals, the LDF recordings were significantly higher than placebo ($P<0.05$) and controls ($P<0.001$) on day 6 after implantation. Similarly, the NIRS recordings in bFGF-stimulated animals were significantly higher than placebo ($P<0.05$) and controls ($P<0.001$) on day 7 after implantation (Fig. 3).

In TNP-470-treated bFGF-stimulated animals, LDF ($P<0.05$) and NIRS ($P<0.05$) recordings were significantly lower compared with untreated bFGF-stimulated animals. Likewise, in dexamethasone-treated bFGF-stimulated animals, LDF ($P<0.001$) and NIRS ($P<0.001$) recordings were significantly lower compared with untreated bFGF stimulated animals (Fig. 4). The systemic effects of TNP-470 and dexamethasone did not influence the LDF and NIRS recordings, since there were no significant differences in the LDF and NIRS signals obtained above the unstimulated contralateral hind legs between the treated and untreated groups of animals.

4. Discussion

Our assumption was that focally-induced angiogenesis leads to a local increase in perfusion and haemoglobin concentration, as a result of an increase in vessel density. The exact relationship between vessel growth, perfusion and haemoglobin concentration is more complex, and not fully discernible, but similar assumptions have previously led to measurements of perfusion and haemoglobin concentration as indices of angiogenesis, e.g. ^{133}xenon-clearance from s.c. implanted polyester sponges in rats, LDF on bFGF stimulated regeneration

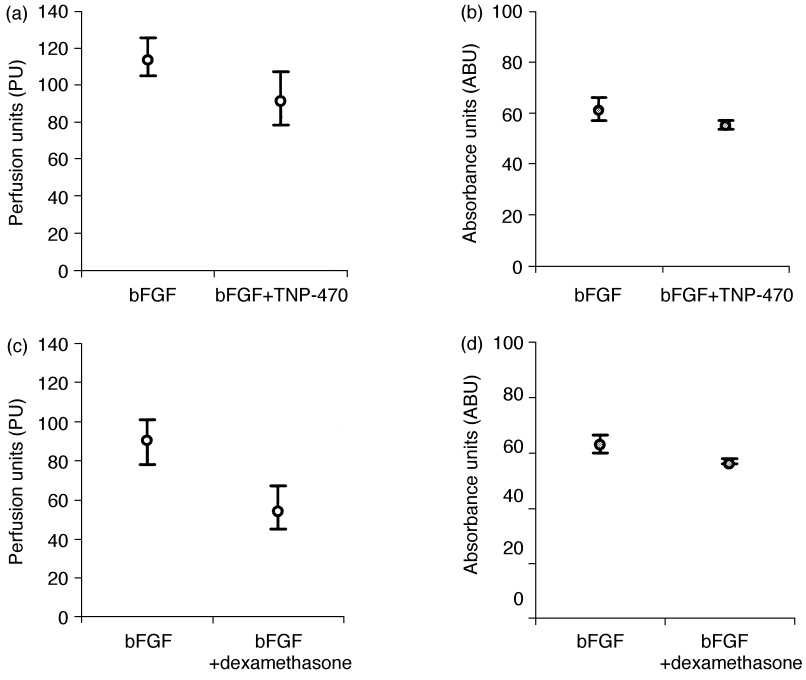

Fig. 4. Anti-angiogenic activity. (a + b) After treatment with TNP-470 (7 mg/kg every day (q.d)×7, $n=13$), LDF ($P<0.05$) and NIRS ($P<0.05$) recordings were significantly lower compared with untreated bFGF-stimulated animals ($n=14$). (c + d) After treatment with dexamethasone (10 mg/kg q.d.×7, $n=12$), LDF ($P<0.001$) and NIRS ($P<0.001$) recordings were significantly lower compared with untreated bFGF-stimulated animals ($n=14$). LDF recordings were performed on day 6, whereas NIRS recordings were performed on day 7 after implantation. Median values and interquartile range.

of tissue grafts in rats, and determination of haemoglobin contents in gel matrixes and sponges containing angiogenic peptides [5,16–18].

In the present study, we measured perfusion by LDF and haemoglobin concentration by NIRS on bFGF-stimulated hind legs of the nude mouse. The results showed that both LDF and NIRS recordings were significantly higher in tissue exposed to angiogenic (bFGF) stimulation than placebo and controls, indicating that LDF as well as NIRS can determine angiogenic activity. The fact that the placebo implants also increased LDF and NIRS recordings significantly from controls demonstrates that the induced angiogenesis was partially caused by a foreign body reaction to the pellet, inducing secondary angiogenesis. This was confirmed by histological examination. An unspecific inflammatory host response to any tissue trauma can interact with and contribute to the angiogenic cascade, which should be taken into consideration with all *in vivo* preparations.

Significantly lower LDF and NIRS recordings in bFGF-stimulated animals treated with the anti-angiogenic compounds TNP-470 and dexamethasone compared with untreated animals, proved that LDF and NIRS can detect the anti-angiogenic activity of a specific agent, as well as an unspecific anti-inflammatory agent.

Dexamethasone reduces the inflammatory response, including the early hyperemic phase, so in theory, this effect might add to what we interpret as an anti-angiogenic response. Fortunately we can exclude this effect on our recordings since we have observed that the hyperemic phase is over within 3 days after implantation. At that time point, an early increase in both LDF and NIRS readings has disappeared. We used recordings from the 6–7 day interval for the evaluation of angiogenic activity.

In order to compensate for the spatial variation of the individual LDF and NIRS recordings, we used LDF and NIRS probes collecting from several fibres simultaneously. Recordings from larger tissue volumes obtained by several fibres increase the number of contributing capillaries and thereby reduce the variation and improve reproducibility [19]. Still, the CV in the hind leg skin of the nude mouse measured by LDF was 20%, concordant with results obtained by others in the skin of hairless mice (CV 17%) [20] and the skin of humans (CV 25%) [21]. The CV in the skin of the nude mouse measured by NIRS was much lower (3%), but this value was not directly comparable to the CV measured by LDF because of different calibration.

A major advantage of the present method is the technical simplicity and minimal animal stress. In contrast, the corneal assay [22] in which chemical material is implanted intracorneally is more complicated in mice and may raise concerns from an animal welfare perspective, due to the ophthalmic involvement. Another advantage of the method is that LDF and NIRS permit non-invasive and non-destructive measurements of perfusion and haemoglobin concentration in animals over a long period of weeks or months. This is in contrast to assays, in which each preparation is scored terminally, e.g. histological examination of vascularisation into biocompatible implants [17].

The LDF and NIRS techniques as applied here are topologically limited to the skin or the s.c. space, because both the NIRS reflectance measurements and, especially, LDF have limited measuring depth [3]. In studies of anatomical sites, organs or tumours, not directly accessible by the probe, specifically designed NIRS techniques or more invasive procedures would be necessary. Their combined lack of hair and pigmentation makes the homozygotous nudes of albino background extraordinary well suited for this particular type of study. In our experience, the reaction to recent shaving of haired animals induces bias in the measurements and so does the uneven distribution of pigments in the skin of other strains. Other anatomical regions, e.g. tumours, in animals or humans represent different situations, which need to be evaluated separately.

An anti-angiogenic activity significantly lower than that of TNP-470 in the present dosage will not be picked up by the present techniques, since the sensitivity is limited by the variation in the LDF and NIRS recordings. Testing of new compounds should include a standard treatment, which defines the threshold level of anti-angiogenic activity to elicit further interest.

At present, the preparation has been developed for studies using bFGF-containing implants only. Other angiogenic inducers can be used as well. In current studies the LDF and NIRS components are incorporated into the same probe, and the methods are being adapted to study angiogenic and anti-angiogenic activity in tumorous tissues *in vivo*.

In conclusion, early *in vivo* bFGF-induced angiogenesis could be estimated quantitatively by LDF and NIRS. Furthermore, the anti-angiogenic activity of TNP-470 and dexamethasone could be evaluated simply and quantitatively. The present method appears to be useful for evaluation of the *in vivo* activity of new angiogenic inhibitors under development as candidate anticancer agents.

Acknowledgements

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