Physiologically, angiogenesis (i.e. the formation of new capillary vessels), is an important event in embryonic development and in the adult female reproductive cycle (Gospodarowicz & Thakral, 1978). Under pathological conditions neovascularisation occurs during the wound healing process (Knighton, 1981) and in a variety of diseases ranging from diabetic retinopathy to psoriasis and several types of chronic inflammations (Goldie, 1969). It has also been shown that the process of solid tumours growth is angiogenesis-dependent (Gullino, 1978; Folkman, 1990). Several substances of different chemical nature and cellular origin, including growth factors produced by the neoplastic cells themselves, have been described to be involved in tumour-induced neoangiogenesis (Shing et al., 1985; Folkman & Klagsbrun, 1987) by directly and/or indirectly stimulating endothelial cells proliferation and/or migration (Ausprunk & Folkman, 1977). One of the better characterised among such angiogenic factors is basic Fibroblast Growth Factor (bFGF) (Rifkin & Moscatelli, 1989), whose presence in a large number of normal and malignant cells is well established, and that has been implicated as a major contributing factor in both physiological and pathological neovessel formation (Folkman et al., 1988; Klagsbrun et al., 1986; Thompson et al., 1987; Hayek et al., 1987). Since solid tumour growth and progression are strictly dependent from neovessel formation (Folkman et al., 1989; Brem et al., 1977) interfering with this process by counteracting the effect of angiogenic growth factors could represent a novel and selective therapeutic approach to malignancy.

Suramin, a polysulphonated trypan red derivative used in the past as antitrypanosomnic agent (Hawking et al., 1987), has recently generated interest as an antineoplastic agent (Stein et al., 1989; Myers et al., 1990; La Rocca et al., 1990). Although it seems likely that other biological activities of suramin also participate in the antitumoural effect of this compound (Stein et al., 1989) it has been advanced that an important role is played by its capacity to interfere with the activity of various growth factors. In fact, suramin has been reported to inhibit in vitro the binding of IGF, EGF, PDGF, IL2, TGFβ and bFGF (Pollock & Richard, 1990; Hosang, 1985; Mills et al., 1990) to their cell surface receptors through direct complexation of the growth factors and/or via a modification of the cell receptor (Coffey et al., 1987). This activity could explain suramin inhibition of the in vitro growth of a number of cell lines (Spiegelman et al., 1987; Pienta et al., 1991; Kim et al., 1991).

In this study we report that suramin is able to inhibit in vivo bFGF-induced angiogenesis, and is active in a bFGF-transducer tumour model.

Materials and methods

Chemicals and reagents

Clinical grade, endotoxin-free human recombinant basic Fibroblast Growth Factor (bFGF) was produced in the laboratories of Farmitalia-Carlo Erba (Milano, Italy). Working solutions of bFGF were prepared from sterile aliquots of a frozen bulk solution stored at -80°C; aliquots were thawed and diluted with PBS (Gibco, Grand Island, NY) containing 0.1% bovine serum albumin (BSA) (Sigma, St Louis, MO) immediately prior to use. Porcine intestinal mucosa and bovine kidney heparan sulphate were obtained by Sigma Chemical Co. (St Louis, MO). Suramin (Germanin, Bayer, Germany) was kindly provided by Dr E. Cvikovic (Institut Gustav Roussy, Paris, France), and dissolved in water immediately prior to use.

Animals and tumours

Young adult female C3H/HeN and C57B1/6 mice (Charles River Italy, Calco, CO) weighing 23-25 g, were used. Animals were housed in plastic cages in temperature and humidity controlled conditions; food and water were available ad libitum and a 12 h light/dark schedule was maintained. When necessary mice were anaesthetised by the i.p. injection of chloral hydrate at a dose of 0.5 mg g⁻¹ of body weight. The transplantable M5076 murine reticulosarcoma was originally obtained from the National Cancer Institute (Frederick, MD) and maintained as previously described (Talmadge et al., 1981).

Five mice/group for sponge implantation experiments and ten mice/group for tumour growth assay were used.

bFGF angiogenesis assay

Gelatin sponges (Gelfoam Upjohn, Kalamazoo, MI) cut into strips (approximately 7 by 10 by 20 mm) under sterile conditions were loaded with 0.25 ml of a PBS/BSA 0.1% solution supplemented or not with the desired concentrations of bFGF. A bFGF-heparan or bFGF-heparin sulphate complex was prepared by coincubating bFGF and Heparin at a 1:1 ratio or, for heparin sulphate at a 1:10 ratio, for 2 h at 4°C in PBS-BSA 0.1%. Sponges were then handled as described above for bFGF alone. Using aseptic techniques, a 1 cm
dorsal skin incision was made proximately to the base of the tail and by gentle dissection with forceps a subcutaneous pouch fashioned 2–3 cm cephalad to the incision. After implantation of the sponge into the s.c. pouch, the skin was sutured. At different times intervals after implantation, mice were sacrificed, the sponges extracted and prepared for visual and histological examinations. Macroscopic angiogenesis was scored on a 0 (absence of neovessel formation) to 5 scale taking into account the number, shape and size of the newly formed blood vessels. Evaluations were made by a single observer in a blind manner. For histological examination, sponges removed from the animals were fixed overnight in neutral buffered formalin; 2–5 μm sections cut crosswise to the sponge center were stained with hematoxylin and eosin. The content of hemoglobin (Hb) extracted from individual sponges was also measured as a parameter of vascularisation of the implant. Hb was extracted from sponges by 4 h incubation in 0.1 M ammonia solution, and measured using a commercial colorimetric assay kit (Merck, Germany).

**M5076 Tumour-induced angiogenesis assay**

For experiments on tumour-induced angiogenesis, sponges were loaded with 0.25 ml of a cell suspension containing 5 × 10⁴ viable M5076 tumour cells in RPMI 1640 medium. Tumour cells were obtained from *in vitro* cultures and their viability was routinely above 90% as evaluated via trypan blue exclusion.

**Tumour growth assay**

The tumour inoculum was of 5 × 10⁵ cells injected s.c. Tumours diameters were measured twice weekly with calipers the tumour weight was estimated as the length × (width)² of each tumour mass × 0.5 (Geran *et al.*, 1972).

Every experiment was repeated at least three times.

**Results**

**bFGF-induced angiogenesis**

bFGF-induced angiogenesis was studied using subcutaneously implanted gelatin sponges loaded with concentrations of bFGF ranging from 0.25 to 10 μg/sponge. A first series of experiments was performed to define both the bFGF dose and the observation time for optimal assessment of the angiogenic response in this model. Table I shows the dose-dependent angiogenic effect of bFGF on day 14 after sponge implant, expressed in terms of angiogenic score. In animals bearing bFGF-treated sponges, several newly-formed blood vessels were observed to infiltrate the sponges and neovascularisation was maximal on 12–15 days from implant. Thereafter, vascularisation gradually decreased, together with a partial reabsorption of the sponge that occurred by day 30–40. The maximal angiogenic response was obtained with 10 μg bFGF, whereas a dose of 0.25 μg was inactive. Control sponges without bFGF were not infiltrated with blood vessel and their content of Hb was irrelevant. Visual evaluation was confirmed by the histological examination (Figure 1). In fact, blood-containing newly formed capillaries were observed in the bFGF-treated sponges together with centripetal infiltration of different cell types, predominantly neutrophils, macrophages and fibroblasts (Figure 1b). In control sponges no newly-formed capillaries were detected and only a sparse cellular infiltrate at the periphery of the implant was observed (Figure 1a). The angiogenic response was quantified also by assessing the Hb content of sponges (Figure 2a). The Hb content in sponges treated with 10 μg of bFGF was 3–4-fold higher than that of untreated controls; Hb levels were clearly lower in the sponges loaded with 2.5 μg of bFGF, whereas in sponges treated with 0.25 μg of bFGF the values were comparable to those of control implants.

Since the angiogenic response was most evident at the dose of 10 μg of bFGF on day 14 after sponge implant, these conditions were chosen for all further experiments.

The effect of suramin in this model was evaluated by treating mice with the drug, i.v. 1 day after implantation of sponges preloaded with 10 μg of bFGF. At 200 mg kg⁻¹ suramin (i.e. the highest drug dose not associated with acute

**Table I** Angiogenic response induced by bFGF-sponges in mice and the effect of Suramin treatment

| bFGF/μg/sponge | Suramin treatment mg kg⁻¹ | Day | Angiogenic score |
|---------------|--------------------------|-----|-----------------|
| bFGF 10       | –                        | –   | ++              |
| bFGF 2.5      | –                        | –   | ++              |
| bFGF 0.25     | –                        | –   | 0               |
| bFGF 100      | 100                      | +   | ++              |
| bFGF 10       | 100                      | +  | ++              |

Mice bearing bFGF-treated sponges were sacrificed 2 weeks after the implants to evaluate angiogenesis. Suramin was given i.v.

![Figure 1](image_url) Hematoxylin eosin stained cross-sections of a, control; b, bFGF-loaded (10 μg) gelfoam sponges and c, bFGF-sponges from mice treated with suramin (200 mg kg⁻¹ on day 1). Figures refer to sponges obtained 14 days after implant.
with heparin was complexed with bFGF. The complex induced angiogenic activity, as confirmed by histological examination, neovessel formation being negligible and cellular infiltration minimal (Figure 1c). The very low Hb content of sponges after suramin treatment further confirmed the absence of angiogenesis (Figure 2a). Conversely, suramin dose of 100 mg kg\(^{-1}\) given at day 1, or a dose of 200 mg kg\(^{-1}\) administered at day 7, did not significantly affect neovascularisation in sponges (Table I) as evaluated histologically or via the Hb content.

bFGF has been demonstrated to interact with heparin and heparan sulphate forming a complex reported to be more resistant than bFGF alone to proteolytic degradation (Saksela et al., 1988; Flaumenhaft et al., 1990). Table II shows the angiogenic response elicited in sponges pre-loaded with bFGF complexed with heparin in a ratio of 1:1. Whereas heparin alone up to 10 μg/sponge had no detectable angiogenic activity, sponges loaded with a bFGF-heparin complex induced a much higher angiogenic response in comparison to bFGF alone. Angiogenesis with bFGF-heparin complex was dose dependent in the tested range of 2.5 to 10 μg/sponge. Sponges loaded with the bFGF-heparin complex showed at the dose of 10 μg of 2-fold higher Hb content compared to that observed in sponges loaded with bFGF alone, whereas in sponges loaded with heparin only no Hb was detected (Figure 2b).

In contrast to what observed with sponges loaded with bFGF alone, suramin administered i.v. at 200 mg kg\(^{-1}\) at day 1 was unable to inhibit neovascularisation induced by bFGF-heparin sponges. In fact the angiogenic score (Table II) and the Hb content of sponges (Figure 2b) were not significantly modified in comparison to controls.

### Table II: Effect of suramin on angiogenesis induced by sponges loaded with complex bFGF-Heparin

| bFGF/sponge μg/sponge | Suramin treatment mg kg\(^{-1}\) day | Angiogenic score |
|-----------------------|-----------------------------------|-----------------|
| bFGF                  | 10                                | ++ +            |
| bFGF                  | 10                                | + +             |
| Heparin               | 10                                | + + + +         |
| bFGF-Heparin          | 10                                | + + + + +       |
| bFGF-Heparin          | 10                                | + + + + + +     |
| bFGF-Heparin          | 2.5                               | + + + + + +     |
| bFGF-Heparin          | 2.5                               | + + + + + +     |

Mice bearing bFGF-treated sponges were sacrificed 2 weeks after the implant. Suramin was given i.v.

#### Tumour-induced angiogenesis

Preliminary experiments in this laboratory had shown that conditioned medium from cultures of M5076 cells contained measurable amounts of bFGF and that it was consistently angiogenic in the rabbit cornea assay. For these reasons the M5076 tumour was chosen for in vivo studies to investigate the possible role of bFGF in tumour angiogenesis and to evaluate the activity of suramin on this process.

In preliminary experiments, different numbers of M5076 tumour cells (from 10^4 to 10^5/sponge) were entrapped in gelfoam sponges and then implanted subcutaneously and angiogenesis was evaluated at different times after implant. An inoculum of 5 x 10^4 cells/sponge was chosen for the subsequent experiments, since it resulted in a clear angiogenic response detectable already at 5–7 days from implantation and that was maximal on 10–15 days (Figure 3a). Newly formed blood vessels of various size and length were observed within the sponge containing the entrapped tumour cells. Administration of a single dose of 200 mg kg\(^{-1}\) i.v. suramin 1 day after sponge implantation significantly delayed M5076 cells induced neovascularisation (Figure 3b). In fact, after suramin treatment, an histologically detectable angiogenesis appeared only 12–15 days from implant, whereas in untreated control animals it was already evident at day 5 and maximal on day 15.

The effect of suramin treatment on the in vivo growth of the M5076 tumour was then investigated and Figure 4a shows the results of different treatment schedules with this drug. Single suramin doses of 200 and 150 mg kg\(^{-1}\) administered i.v. 24 h after s.c. tumour transplant were able to reduce dose-dependently the growth rate of the tumour, whereas 100 mg kg\(^{-1}\) had no significant effect (not shown). Suramin was active only when administered in the first days after tumour cell transplantation and in fact, no tumour growth inhibition was observed when 200 mg kg\(^{-1}\) of suramin was injected on day 10, a time when the tumour was already palpable.

The growth of the M5076 tumour was enhanced by the administration of bFGF. In fact, peritumoral injections with 5 μg of bFGF given daily from day 1 after tumour transplant to day 7, significantly increased tumour growth (Figure 4b). In addition, the growth inhibitory effect of suramin was almost completely counteracted by topical bFGF treatment. In fact, after 20–24 days from the implantation, tumours in animals treated with suramin and peritumour bFGF reached the same size as untreated controls.

#### Discussion

The involvement of bFGF in the angiogenic process is well documented by studies showing bFGF stimulation of endothelial cell proliferation and motility in vitro (Folkman et al., 1988), induction of angiogenesis in the chick embryo chorioallantoic membrane, and in the rabbit cornea assay
Figure 3 Tumour-induced angiogenesis in sponges loaded with M5076 cells. An evident angiogenic response is visible after 15 days from implant of the sponge loaded with M5076 cells. a. Suramin treatment inhibit tumour-induced neovascularisation b.

(Folkman & Klagsbrun, 1987). Mice bearing s.c. gelfoam sponges loaded with bFGF developed a clear angiogenic response within the implant. This response was dramatically reduced by the systemic administration of suramin as determined by histological and biochemical parameters. In this model suramin exerted maximal activity if administered on the first day after implant, whereas no effect was seen if the compound was given on day 7 at the dose fully inhibitory on day 1. Taking also in account that suramin is endowed with an extremely long half-life in the body (Stein et al., 1989), these findings indicate that in this experimental conditions suramin interferes preferentially with the initial phases of the angiogenetic process, whereas it lost effectiveness when the process was already established. Previous studies (Coffey et al., 1987) have shown that suramin by binding bFGF prevented the interaction of this growth factor with its receptors. Moreover, in our hands suramin was not cytotoxic in vitro for resting or proliferating endothelial cells even after long (96 h) exposure times. These observations suggest that also in the in vivo experimental model employed endothelial cells were not directly affected in their viability by suramin. The finding that suramin was only active in the early phase of angiogenesis is a further, albeit indirect, support for the contention that in the bFGF-sponge model, suramin activity derives from its capacity to bind and inactivate bFGF, a key factors in stimulating endothelial cells multiplication and thus in angiogenesis.

In physiological conditions bFGF present in tissues is essentially bound to heparan sulphates associated to the extracellular matrix (Folkman et al., 1988), and is released complexed to heparan sulphate after degradation of the extracellular matrix (Saksela et al., 1988). This complex has been shown to be more resistant to protease degradation and to better diffuse in the extracellular environment than free bFGF (Flaumenhaft et al., 1990). In line with the above observations, the angiogenetic response elicited by bFGF when complexed to heparin or heparan sulphate was much higher than seen in sponges with free bFGF. Suramin was inactive against the bFGF-heparin complex suggesting that heparin might compete for the binding to bFGF, with a much higher affinity than suramin. Importantly however, while the biological functions of bFGF are maintained and enhanced after binding to heparin, the binding with suramin impairs bFGF activity.
Since it has been advanced that bFGF plays a key role also in tumour induced neovascularisation (Folkman et al., 1988; Klagsbrun et al., 1986), and, as recently described (Hori et al., 1991; Gross et al., 1990), in tumour growth, it was of interest to investigate suramin in a simplified model of tumour-associated angiogenesis as that represented by M5076 cells entrapped in gel foam sponges. Results obtained were similar to those seen with bFGF sponges since suramin was effective only when given within 24–48 h after implantation, i.e. during the early stages of the tumour growth.

Suramin was also able to reduce the growth of M5076 tumour transplanted subcutaneously, with the maximal activity seen administering the compound on the first day after tumour transplantation. In this sytem, repeated peritumoural injections of bFGF increased the tumour growth rate, and significantly reduced the tumour-growth inhibitory activity of suramin. Since suramin up to the concentration of 150 μg ml⁻¹ (data not shown) was not cytotoxic to cultured M5076 cells, the hypothesis can be advanced that suramin delays M5076 tumour growth not via a direct cytotoxicity on neoplastic cells, but indirectly through interference with neovessel formation elicited by tumour-produced bFGF. It should be noted however that the role of other growth factors involved in angiogenesis (e.g. PDGF, TGFβ, etc.), was not evaluated in this study; accordingly it cannot be excluded that suramin also interferes with their activity in tumour growth.

In conclusion, these results provide further direct support to the conclusion that a major component in the antineoplastic activity of suramin may be an interference with neovascularisation induced by growth factors such as bFGF, produced by the neoplastic cells. Since suramin administration is associated with important toxicities in both animals and humans (Stein et al., 1989; La Rocca et al., 1990), the identification of novel molecules capable of interfering with tumour-induced angiogenesis, but possessing a more favourable therapeutic index, could open alternative approaches to the treatment of solid neoplasms.

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