Effective Cancer Targeting Using an Anti-tumor Tissue Vascular Endothelium-specific Monoclonal Antibody (TES-23)

Yukiko Wakai,1 Junji Matsui,1 Keiichi Koizumi,1 Shin-ichi Tsunoda,1 Hiroo Makimoto,1 Iwao Ohizumi,2 Kenji Taniguchi,2 Shin-ichi Kahi,2 Hiroyuki Saito,2 Naoki Uotoguchi,3 Yasuo Tsutsumi,1 Shinsaku Nakagawa,1 Yoshiyuki Ohsugi2 and Tadanori Mayumi1, 4

1Department of Biopharmaceutics, School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, 2Department of Cancer Research, Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-0038 and 3Showa College of Pharmaceutical Sciences, 3-3165 Higashi Tamagawa Gakuen, Machida, Tokyo 194-0041

Immuconjugate targeting of solid tumors has not been routinely successful because the endothelial cells of blood vessels act as a physical barrier against the transport of macromolecules, such as antibodies. In the present study, we attempted to achieve tumor vascular targeting with an anti-tumor tissue endothelium-specific monoclonal antibody (TES-23). TES-23, an IgG1 monoclonal antibody raised against rat KMT-17 fibrosarcoma-derived endothelial cells, was covalently conjugated with neocarzinostatin (NCS) in a previous study. The TES-23-NCS conjugate induced tumor hemorrhagic necrosis, and showed marked anti-tumor effects against rat KMT-17 fibrosarcoma. This result prompted us to investigate whether this approach would be applicable to various other types of solid tumors. One hour after injection of 125I-labeled TES-23 into BALB/c mice bearing Meth-A fibrosarcoma and Colon 26 adenocarcinoma, the tumor accumulation of TES-23 was greater than that of the control IgG. In the present study, we report the anti-tumor effects of this monoclonal antibody in mice bearing Meth-A fibrosarcoma. Mice treated with the immunoconjugate showed improved survival with no side effects. This result indicates that common antigens may be found in different kinds of tumor endothelial cells, and that TES-23 might recognize these antigens.

Key words: Tumor vascular endothelium — Immunoconjugate — Cancer targeting therapy — Immunoconjugates

Targeting therapy for cancer is necessary because of the severe side effects of anticancer drugs. Although it is attractive to use immunoconjugates of a monoclonal antibody and a drug for targeting therapy of cancer, the use of monoclonal antibodies against tumor cells has been ineffective due to poor penetration into tumor tissue.1–3) Furthermore, targeting of tumor cells requires monoclonal antibodies to the specific antigens expressed by each type of tumor cell, but the antigens of tumor cells are heterogeneous.4–6) As a step towards overcoming this problem, we previously reported the use of monoclonal antibodies against tumor-tissue endothelial cells. Tumor growth is dependent on angiogenesis to supply nutrients and oxygen.7) Damage to the tumor vasculature induces tumor regression.8) It has been demonstrated that the vasculature of solid tumors is a possible target for an immunotoxin.9) Vascular endothelial cells of different types of tumors generally have similar characteristics, such as high penetration10–12) and high TNF (tumor necrosis factor)-sensitivity.13) Thus, it is possible that common molecules are expressed by tumor tissue endothelial cells of various types of solid tumors. Furthermore, as tumor vascular endothelial cells are in intimate contact with the blood stream, targeting therapy using monoclonal antibodies against molecules expressed in tumor tissue endothelial cells may permit antibodies to recognize the target cells directly without a physical barrier.

For such targeting therapy to succeed, antibodies that are specific for tumor endothelial cells are required. However, no antibodies that recognize only tumor vasculature have been reported, and tumor tissue endothelial cells have not yet been isolated and cultured. To prepare such antibodies, we previously isolated and cultivated endothelial cells (TEC) from KMT-17 fibrosarcoma, and examined their properties in culture.14) We then prepared the monoclonal antibody TES-23 against tumor tissue endothelial cells by immunization of mice with TEC.15) Next, we immunoconjugated TES-23 with neocarzinostatin (NCS).16)

In the present report, we describe the anti-tumor effects of the immunoconjugate in the treatment of mice bearing Meth-A fibrosarcoma as part of a program to examine the applicability of this approach to different types of tumors. Mice treated with the immunoconjugate showed improved survival with no side effects. One hour after injection of

4To whom correspondence should be addressed.
E-mail: mayumi@phs.osaka-u.ac.jp
125I-labeled TES-23 into BALB/c mice bearing Meth-A fibrosarcoma and Colon 26 adenocarcinoma, the tumor accumulation of TES-23 was greater than that of the IgG control.

**MATERIALS AND METHODS**

**Animals and cells** BALB/c mice (female, 5 weeks old) were purchased from Japan SLC (Hamamatsu). All experimental protocols involving animals complied with our Institutional Guide for the Care and Use of Laboratory Animals.

**Monoclonal antibodies** TES-23 (tumor endothelium-specific mouse monoclonal antibody) was prepared as described previously,14, 15) Mopc was prepared from the mouse plasmacytoma MOPC-31C (ATCC. CCL-130) as a nonspecific control antibody. Antibodies from ascites fluid of hybridoma-implanted BALB/c mice were purified using a Protein A column (Bio Rad Laboratory, Richmond, CA).

**Preparation of antibody-NCS conjugates** Cross-linking reaction was performed as previously described, with slight modifications.13) The antibodies (5–8 mg/ml) were modified with 3-(2-pyridyldithio)propionyl hydrazide (PDPH; Pierce), a carbohydrate-directed heterobifunctional cross-linking reagent, and then oxidized in 0.1 M acetate buffer (pH 5.5) containing 1 mM sodium periodate and 3 mM PDPH for 15 min at 0°C. The reaction mixture was subsequently passed through an Econo-Pac™ 10DG (Bio Rad Laboratory) equilibrated with 0.1 M phosphate buffer at pH 8.0. A ten-fold molar excess of PDPH was added to the oxidized antibodies (3–5 mg/ml) three times every 2 h at 25°C. After incubation for 12 h, residual PDPH was removed by gel filtration using an Econo-Pac™ 10DG equilibrated with 0.1 M phosphate buffer at pH 7.2. The degree of substitution with PDPH was determined as previously reported.16) The conjugation rate was calculated to be approximately 4.5 mol of PDPH per mol of antibody, NCS, kindly provided by Dr. M. Hirama (Tohoku University, Sendai), was incubated with a ten-fold molar excess of 2-iminothiolane (IT; Pierce) in 0.1 M phosphate buffer, pH 8.0, for 45 min at 25°C in the dark. NCS with an introduced thiol group (HS-NCS) was purified using an Econo-Pac™ 10DG equilibrated with 0.1 M phosphate buffer at pH 7.2. Finally, PDPH-derivatized antibodies were mixed with a three-fold molar excess of HS-NCS overnight at 25°C in the dark. The reaction mixture was then applied to a TSK gel G3000SWXL column ( Tosoh Co., Ltd., Tokyo) equilibrated with 0.1 M phosphate buffer at pH 7.2. Fractions containing conjugates were collected and stored at −20°C in the dark. The conjugation rate was estimated to be approximately 2 mol of NCS per 1 mol of antibody.

**Anti-tumor activity of TES-23-NCS** Meth-A fibrosarcoma cells were cultured by intraperitoneal passage in BALB/c mice. Meth-A cells (5×10⁵ cells/mouse) were implanted intradermally into the abdomens of 5-week-old female BALB/c mice. Groups of four animals were injected intravenously with TES-23-NCS conjugate (TES-23: 320 µg/kg, NCS: 50 µg/kg, or TES-23: 107 µg/kg, NCS: 17 µg/kg), TES-23-NCS conjugate (TES-23: 107 µg/kg, NCS: 17 µg/kg) plus TES-23 (1070 µg/kg), MOPC-NCS conjugate (MOPC-31C: 320 µg/kg, NCS: 50 µg/kg, or MOPC-31C: 107 µg/kg, NCS: 17 µg/kg), TES-23 (320 µg/kg, NCS 500 µg/kg or 50 µg/kg), TES-23 (320 µg/kg) plus NCS (50 µg/kg), or phosphate buffer at pH 7.2, respectively, on days 9, 11 and 13 following tumor inoculation. The anti-tumor effects against Meth-A fibrosarcoma were expressed in terms of mean tumor volume and life span. Tumor volume was calculated using the formula \(1/2ab^2\) (\(a\): long axis, \(b\): short axis). Body weight was measured simultaneously. Blood samples were taken from the tail vein, and the hematocyte count was measured using a Sysmex platelet counter.

**Tissue distribution studies** Meth-A fibrosarcoma cells were cultured by intraperitoneal passage in BALB/c mice. Meth-A fibrosarcoma cells (3×10⁴ cells/mouse) were implanted intradermally into the abdomens of 5-week-old female BALB/c mice. Colon 26 adenocarcinoma cells were subcultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), and then implanted intradermally into the abdomens of 5-week-old female BALB/c mice (5×10⁵ cells/mouse). TES-23 and MOPC-31C were radio-labeled with 125I using Iodogen.18) Nine days later, groups of five animals bearing Meth-A fibrosarcoma or Colon 26 adenocarcinoma were injected intravenously with 125I-TES-23 or 125I-MOPC-31C at 20 ng/mouse (200 000 cpm/mouse), and the mice were sacrificed 1 h after injection. Tissue was then excised, the weight was recorded, and radioactivity was counted using a γ counter.

**RESULTS**

Anti-tumor effects in mice bearing Meth-A fibrosarcoma The anti-tumor effects of TES-23-NCS conjugate against Meth-A fibrosarcoma are shown in Fig. 1. Groups of four mice bearing tumors of approximately 0.1 cm³ in volume were injected intravenously with test samples on day 9 following tumor inoculation. Drug administration was performed three times every two days. TES-23-NCS conjugate (TES-23: 320 µg/kg, NCS: 50 µg/kg) showed marked anti-tumor activity (Fig. 1). Notably, complete regression was observed in three of the four mice in this group (Table I). The other TES-23-NCS conjugate (TES-23: 107 µg/kg, NCS: 17 µg/kg) also significantly suppressed solid tumor formation (Fig. 1), and complete regression was observed in two of the four mice in this group (Table I). In contrast, NCS alone did not inhibit
tumor growth at a dose of 50 or 500 µg/kg. The mean volume of tumors in mice treated with TES-23-NCS conjugate (50 µg/kg) was less than that in animals treated with free NCS (500 µg/kg). On the other hand, TES-23 alone had no effect on tumor growth, and mixtures of NCS and TES-23 were no more effective when tested at the same dose (Fig. 1a). Moreover, when a high dose of TES-23 alone (1070 µg/kg) was injected intravenously into the animals, no anti-tumor activity was observed, although TES-23-NCS conjugate induced complete regression (TES-23: 107 µg/kg, NCS: 17 µg/kg). Mouse IgG conjugate (MOPC-NCS conjugate) showed no anti-tumor effect.

![Fig. 1. Anti-tumor effects of TES-23-NCS on Meth-A solid tumor in BALB/c mice.](image)

Table I. Antitumor Effects of TES-23-NCS in Terms of Survival Days after Meth-A Tumor Inoculation

| Injection dose (µg/kg) | Survival timea (days) | Completeb |
|-----------------------|-----------------------|-----------|
| Ab                    | NCS                   |           |
| Control               | —                     | 53±6      |
| TES-23-NCS 320        | 50                    | 103±19    |
| 107                   | 17                    | 95±17     |
| TES-23-NCS 320        | 50                    | 46±5      |
| + TES-23 (1070 µg/kg) |                       |           |
| MOPC-NCS 320          | 50                    | 50±3      |
| 107                   | 17                    | 45±3      |
| NCS                   | —                     | 63±25     |
| 107                   | 17                    | 49±6      |
| TES-23 320            | —                     | 45±4      |
| TES-23 + NCS 320      | 50                    | 51±3      |

a) Groups of four mice received each treatment intravenously at days 9, 11, and 13.
b) Days after tumor inoculation (mean±SE).
c) Complete regression was defined as no tumor regrowth for more than 120 days.
d) Significant difference from the control group (P<0.05).
These observations indicate that TES-23-NCS is as effective against Meth-A fibrosarcoma as it is against KMT-17 fibrosarcoma.

### Side effects of TES-23-NCS conjugate

The mean body weight of mice treated with free NCS (500 µg/kg) was significantly lower than that of the control mice. On the other hand, the group treated with TES-23-NCS conjugate showed no reduction in body weight (Fig. 2a). The group treated with free NCS (500 µg/kg) also showed a significant decrease in platelet count, whereas TES-23-NCS conjugate did not induce any platelet reduction (Fig. 2b). No effects were apparent on erythrocyte or leukocyte counts in any of the groups (data not shown).

### Tissue distribution of TES-23

Twenty nanograms of 125I-labeled TES-23 or MOPC-31C, 2x10^5 cpm, was injected intravenously into BALB/c mice. Values shown are averages of five animals sacrificed at 1 h. ■ TES-23, □ MOPC. All data are expressed as mean % of injected dose/g tissue; bars, SE. (Fig. 1b). These observations indicate that TES-23-NCS is as effective against Meth-A fibrosarcoma as it is against KMT-17 fibrosarcoma.

**Side effects of TES-23-NCS conjugate** The mean body weight of mice treated with free NCS (500 µg/kg) was significantly lower than that of the control mice. On the other hand, the group treated with TES-23-NCS conjugate showed no reduction in body weight (Fig. 2a). The group treated with free NCS (500 µg/kg) also showed a significant decrease in platelet count, whereas TES-23-NCS conjugate did not induce any platelet reduction (Fig. 2b). No effects were apparent on erythrocyte or leukocyte counts in any of the groups (data not shown).

**Tissue distribution of TES-23** 125I-Labeled TES-23 and MOPC-31C were injected into BALB/c mice bearing Meth-A fibrosarcoma (Fig. 3). At one hour after administration, the tumor concentrations of TES-23 and MOPC-31C were 111.8±16.4% and 2.1±0.066% of the injected dose/g, respectively. The level of TES-23 was 50-fold greater than that of MOPC-31C. The results of experiments in BALB/c mice bearing Colon 26 adenocarcinoma are shown in Fig. 4. The tumor accumulation of TES-23
Tumor Vascular Targeting

was 20-fold higher than that observed in the control non-immune mouse immunoglobulin group. TES-23 and MOPC-31C were localized at 81.5 ± 9.5% and 3.9 ± 0.47% of the injected dose/g, respectively. No specific distribution of TES-23 was observed in any other tissues in BALB/c mice with Meth-A fibrosarcoma or Colon 26 adenocarcinoma.

DISCUSSION

Although several tumor-associated antigens have been reported, immunoconjugates targeting solid tumors have not been successful from the viewpoint of cancer therapy due to poor penetration of tumor vasculature1–3) and the fact that the antigen expression patterns of malignant cells in tumor tissue are heterogeneous.4, 5) One possible means of selectively targeting tumor vasculature was proposed.8) Damage to tumor vasculature should induce tumor regression. The present report shows that the anti-TEC monoclonal antibody TES-23 targets the vasculature of different types of tumors, suggesting that TES-23 recognizes the same antigen in many kinds of tumor endothelial cells. TES-23-NCS, even at only 17 µg/kg NCS, induced regression of Meth-A fibrosarcoma, whereas 500 µg/kg NCS alone did not (Fig. 1a). The tumor exhibited hemorrhagic necrosis (data not shown), which might be due to the collapse of the endothelial cells of the blood vessels attacked by TES-23-NCS. The addition of a ten-fold excess of free TES-23 resulted in competitive inhibition of the anti-tumor effect of TES-23-NCS conjugates. MOPC-NCS conjugates showed no anti-tumor effect (Fig. 1b).

We found 111.8% of the injected dose of TES-23/g in Meth-A fibrosarcoma (Fig. 3), and in 81.5% in Colon 26 adenocarcinoma (Fig. 4) only 1 h after administration. A recent study indicated that antibodies against vascular endothelial cells rapidly accumulated at a high percentage of the injected dose/g.9) In contrast, an antibody against tumor cells showed only a low level of accumulation in the target tissue, even after 24 h.

TES-23-NCS showed clearer anti-tumor effects against Meth-A fibrosarcoma than against KMT-17 fibrosarcoma. Murine monoclonal antibodies have been reported to react to rat colon tumors more effectively in nude mice than in rats.9) TES-23 did not react with normal tissues of BALB/c mice because it was prepared by immunization of BALB/c mice.15) This antibody may recognize a novel antigen specifically expressed by tumor vascular endothelial cells.

An ideal antigen for vascular targeting would be expressed in tumor endothelial cells at a high ratio, but not in the cells of normal blood vessels. Several tumor vascular endothelial cell markers and antibodies have been reported to date20–23) but no antigens exclusively expressed by tumor endothelial cells have been found. Therefore, TES-23 might be effective for cancer targeting therapy.

Tumor endothelial cells are exposed to a number of tumor fluid factors. These tumor fluid factors induce angiogenesis, and up-regulation of several molecules occurs under these conditions. A number of proteins are specifically induced in human umbilical vein endothelial cells in the presence of tumor-conditioned medium.24) We reported previously that tumor cell-conditioned medium altered some of the characteristics of endothelial cells, such as hyperpermeability.25) This indicates that tumor cells greatly influence endothelial cells, and that many kinds of tumor vasculature show similar characteristics, even though endothelial cells are different from normal cells.

Cancer therapy using monoclonal antibodies that target tumor cells is disadvantageous insofar as the antibodies have difficulty gaining access to the tumor due to the physical barrier of the endothelial cells of the blood vessels. To overcome this disadvantage, we used an anti-tumor tissue endothelial cell monoclonal antibody. As tumor vascular endothelial cells are in intimate contact with the blood stream, it is possible for antibodies to recognize the target cells directly without a physical barrier. Furthermore, vascular targeting is applicable to many different types of solid tumors, because the vasculature generally has similar characteristics, such as high penetrability. Our previous observations suggested the presence of a common antigen in tumor vascular endothel-
lial cells. Therefore, we prepared the monoclonal antibody TES-23 against tumor-derived endothelial cells from rat KMT-17 fibrosarcoma, and conjugated it with NCS. In a recent report, we described the anti-tumor activities of this immunoconjugate as evaluated in rats bearing KMT-17 fibrosarcomas. This led us to investigate whether this approach would also be applicable to other types of solid tumors. Here, we have shown that this immunoconjugate is active in mice bearing Meth-A fibrosarcoma, resulting in long-term survival with no side effects. After one injection of $^{125}$I-labeled TES-23 into BALB/c mice bearing Meth-A fibrosarcoma and Colon 26 adenocarcinoma, the tumor accumulation of TES-23 was greater than that observed in the control nonimmune mouse IgG group.

These results indicate that different types of tumor endothelial cells might express common antigens, and that TES-23 might recognize these antigens. The nature of the antigen remains to be established, though we speculate that it might be related to CD44.

ACKNOWLEDGMENTS

This work was supported in part by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science, and in part by Grants-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, as well as Grants for Research on Health Sciences from the Ministry of Health and Welfare of Japan.

(Received May 26, 2000/Revised August 23, 2000/Accepted September 1, 2000)

REFERENCES

1) Dvorak, H. F., Nagy, J. A. and Dvorak, A. M. Structure of solid tumors and their vasculature: implications for therapy with monoclonal antibodies. Cancer Cells, 3, 77–85 (1991).
2) Zlotecki, R. A., Baxter, L. T., Boucher, Y. and Jain, R. K. Pharmacologic modification of tumor blood flow and interstitial fluid pressure in a human tumor xenograft: network analysis and mechanistic interpretation. Microvasc. Res., 50, 429–443 (1995).
3) Burrows, F. J., Watanabe, Y. and Thorpe, P. E. A murine model for antibody-directed targeting of vascular endothelial cells in solid tumors. Cancer Res., 52, 5954–5962 (1992).
4) Thorpe, P. E., Wallace, P. M., Knowles, P. P., Relf, M. G., Brown, A. N., Watson, G. J., Knyba, R. E., Wawrzynzczak, E. J. and Blakely, D. C. New coupling agents for the synthesis of immunoconjugates containing a hindered disulfide bond with improved stability in vivo. Cancer Res., 47, 5924–5931 (1987).
5) Engert, A., Martin, G., Pfreundschuh, M., Amlot, P., Hsu, S. M., Diehl, V. and Thorpe, P. E. Antitumor effects of ricin A chain immunoconjugates prepared from intact antibodies and Fab' fragments on solid human Hodgkin's disease tumors in mice. Cancer Res., 50, 2929–2935 (1990).
6) Shockley, T. R., Lin, K., Nagy, J. A., Tompkins, R. G., Dvorak, H. F. and Yarmush, M. L. Penetration of tumor tissue by antibodies and other immunoproteins. Ann. NY Acad. Sci., 618, 367–382 (1991).
7) Folkman, J. The role of angiogenesis in tumor growth. Semin. Cancer Biol., 3, 65–71 (1992).
8) Denekamp, J. Vascular attack as a therapeutic strategy for cancer. Cancer Metastasis Rev., 9, 267–282 (1990).
9) Burrows, F. J. and Thorpe, P. E. Vascular targeting—a new approach to the therapy of solid tumors. Pharmacol. Ther., 64, 155–174 (1994).
10) Dvorak, H. F., Nagy, J. A., Dvorak, J. T. and Dvorak, A. M. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am. J. Pathol., 133, 95–109 (1988).
11) Yuan, F., Leunig, M., Berk, D. A. and Jain, R. K. Microvascular permeability of albumin, vascular surface area, and vascular volume measured in human adenocarcinoma LS174T using dorsal chamber in SCID mice. Microvasc. Res., 45, 269–289 (1993).
12) Wu, N. Z., Klitzman, B., Dodge, R. and Dewhirst, M. W. Diminished leukocyte-endothelium interaction in tumor microvessels. Cancer Res., 52, 4265–4268 (1992).
13) Nawroth, P. P., Bank, I., Handley, D., Cassimeris, J., Chess, L. and Stern, D. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J. Exp. Med., 163, 1363–1375 (1986).
14) Utoguchi, N., Dantakean, A., Makimoto, H., Wakai, Y., Tsutsu, Y., Nakagawa, S. and Mayumi, T. Isolation and properties of tumor-derived endothelial cells from rat KMT-17 fibrosarcoma. Jpn. J. Cancer Res., 86, 193–201 (1995).
15) Ohizumi, I., Tsuchida, S., Taniguchi, K., Saito, H., Esaki, K., Koizumi, K., Makimoto, H., Wakai, Y., Matsui, J., Tsutsu, Y., Nakagawa, S., Utoguchi, N., Ohsugi, Y. and Mayumi, T. Monoclonal antibodies recognize antigens expressed on rat tumor vasculature. Int. J. Cancer, 77, 561–566 (1998).
16) Makimoto, H., Koizumi, K., Tsuchida, S., Wakai, Y., Matsui, J., Tsutsu, Y., Nakagawa, S., Ohizumi, I., Taniguchi, K., Saito, H., Utoguchi, N., Ohsugi, Y. and Mayumi, T. Tumor vascular targeting using a tumor-tissue endothelium-specific monoclonal antibody as an effective strategy for cancer chemotherapy. Biochem. Biophys. Res. Commun., 260, 346–350 (1999).
17) Zara, J. J., Wood, R. D., Boon, P., Kim, C. H., Pomato, N., Brederhorst, R. and Vogel, C. W. A carbohydrate-directed heterobifunctional cross-linking reagent for the synthesis of immunoconjugates. Anal. Biochem., 194, 156–162 (1991).
18) Richardson, A. P., Mountford, P. J., Baird, A. C., Heyderman, E., Richardson, T. C. and Coakley, A. J. An improved iodogen method of labelling antibodies with $^{125}$I. Nucl. Med. Commun., 7, 355–362 (1986).

19) Laborda, J., Douillard, J. Y., Lizzio, E. F. and Hoffman, T. Comparative pharmacokinetics of a murine monoclonal antibody to a rat colon tumor in rats and nude mice. Cancer Res., 50, 873–876 (1990).

20) Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushima, H. and Sato, M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene, 5, 519–524 (1990).

21) Millauer, B., Wizigmann, V. S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W. and Ullrich, A. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell, 72, 835–846 (1993).

22) Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A. and Cheresh, D. A. Definition of two angiogenic pathways by distinct alpha v integrins. Science, 270, 1500–1502 (1995).

23) Maunoury, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N., Daly, T. J., Davis, S., Sato, T. N. and Yazopoulos, G. D. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science, 277, 55–60 (1995).

24) Clarke, M. S., Kiff, R. S., Kumar, S., Kumar, P. and West, D. C. The identification of proliferation-related proteins in human endothelial cells as a possible target in tumor therapy. Int. J. Radiat. Biol., 60, 17–23 (1991).

25) Utoguchi, N., Mizuguchi, H., Saeki, K., Ikeda, K., Tsutsumi, Y., Nakagawa, S. and Mayumi, T. Tumor-conditioned medium increases macromolecular permeability of endothelial cell monolayer. Cancer Lett., 89, 7–14 (1995).