Combination of Interferon-β and ACE Inhibitor, Perindopril, Attenuates the Murine Hepatocellular Carcinoma Development and Angiogenesis

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The abbreviations used in this paper: AT-II, angiotensin-II; EC, endothelial cell; HCC, hepatocellular carcinoma; IFN, interferon; PE, perindopril; VEGF, vascular endothelial growth factor

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ABSTRACT

Purpose: **Angiogenesis is now recognized as a crucial step in the development of tumors, including hepatocellular carcinoma (HCC). The aim of this study was to elucidate the combined effect of the clinically used angiotensin I-converting enzyme (ACE) inhibitor, perindopril (PE), and interferon-β (IFN) on the murine HCC development and angiogenesis at clinically comparable low doses.** ***Experimental Design:*** **PE and IFN were administered at doses of 2 mg/kg/day and $1x10^4$ IU/twice a week, respectively.** ***Results:*** **Both PE and IFN significantly suppressed the HCC development and inhibited neovascularization in the tumor, although the effect of low-dose IFN was weaker than that of PE. A combination regimen of PE plus IFN was effective as IFN significantly augmented the tumoricidal effect of PE. These inhibitory effects of PE plus IFN could be detected even on established tumors. The potent angiogenic factor, vascular endothelial growth factor (VEGF), was markedly suppressed by the combined treatment with PE and IFN, whereas these agents resulted in a marked increase of apoptosis in the tumor. The *in vitro* studies exhibited that PE and IFN inhibited the endothelial cell (EC) tubular formation. IFN also suppressed the EC proliferation, whereas neither IFN nor PE**

showed any inhibitory effect on HCC cells proliferation. *Conclusion:* The combination treatment of PE and IFN at clinically comparable low doses could inhibit HCC development and angiogenesis and suppress VEGF as well. Since both agents are widely used in the clinical practice, this combination regimen may represent a potential new strategy for HCC therapy in the future.

INTRODUCTION

Therapies aiming at destruction of the tumor vasculature can achieve rapid regression of experimental tumors, and it has been shown that tumor cell apoptosis is significantly increased by treatment with anti-angiogenic agents (1-3). It has been documented that anti-angiogenic therapy showed less drug resistance than the conventional chemotherapy. With regard to the conventional chemotherapy, drug resistance is encountered in about 30% of all cancer patients. The tumor cells have been shown to readily acquire drug resistance because of their genetic instability, heterogeneity, and high mutation rate, whereas the endothelial cells (EC) are genetically stable and acquire much less drug resistance (4, 5). Accordingly, the anti-angiogenic therapy is under investigation around the world, including the use of gene therapy, anti-angiogenic recombinant proteins, monoclonal antibodies, and various drugs. Although some of these agents are now used in phase I, II , and III clinical trials at certain institutes, no agent is widely available at this time in the clinical practice (6). In of the concept of anti-angiogenesis therapy, long-term administration is required to examine the compound toxicity. One potential alternative strategy may be the use of drugs with anti-angiogenic activity, available in an oral formulation, which are currently administered to patients for treatment of different diseases. Some

of the clinically available compounds, such as thalidomide and penicillamine, have been shown to possess anti-angiogenic activity, and are currently used in clinical trials (6). Long-term administration, however, of these agents sometimes leads to severe side-effects, such as bone marrow suppression.

Recently, a retrospective cohort study on 5207 patients receiving ACE inhibitor or other anti-hypertensive drugs with a 10-year follow-up has showed that the ACE inhibitor decreased incidence of cancer (Glasgow study)(7). Angiotensin-II (AT-II) is an octapeptide produced mainly by proteolytic cleavage of its precursor AT-I by angiotensin-converting enzyme (ACE)(8). It has been shown that AT-II selectively increased the blood vessel flow, and that ACE inhibitor decreased the intratumoral blood flow without affecting the blood flow in the healthy organs (9). AT-II induces angiogenesis in several types of cells, including HCC, and the activity of ACE was used as a tumor marker in the HCC patients (10-12). We previously reported that the clinically used ACE inhibitor, perindopril (PE), possesses a strong anti-angiogenic activity, and that it inhibited the murine HCC growth at the clinically comparable low doses. PE also suppressed the expression of the vascular endothelial growth factor (VEGF), which is known as one of the most potent angiogenic factors, in

the tumor (12).

Interferons (IFNs) are a family of natural glycoproteins initially discovered on their basis of the antiviral activity (13). Now, it is known that IFNs are multifaceted agents for their ability to influence proliferation, differentiation, and the immune system (14). In addition, IFNs also show anti-angiogenic activity both *in vitro* and *in vivo* (15, 16). Frequent systemic administration or gene delivery of IFN exerted a therapeutic effect in some experimental models (17-19). In HCC, it has been shown that high dose and long-term therapy with IFN-α inhibited experimental HCC development, and IFN-β prevented recurrence of HCC in man (20, 21). Most patients, however, receiving high doses of IFNs experience some degree of acute toxicity as well. The most common side effects are flu-like symptoms that include chills, fever, myalgia, and headache. Chronic exposure results in several additional symptoms including fatigue, anorexia, weight loss, dizziness, and some hematological disorders (21, 22). These side effects make long-term administration of high dose of IFNs unlikely to be acceptable.

It has been reported that combination treatment of different anti-angiogenic agents exerted a potent inhibitory effect on tumor development than single agent treatment (6). In the current study, to

evaluate the possible feasibility of future clinical application, we examined the combination effect of PE and IFN-β at clinically comparable low doses on the murine HCC tumor development and angiogenesis, and we attempted to investigate the possible mechanisms involved.

MATERIALS AND METHODS

Compounds and cell lines. Perindopril (PE) and IFN-β (IFN) were generously supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan) and TORAY Industries, Inc. (Tokyo, Japan), respectively. The murine HCC cell line, BNL. 1 ME A.7R.1 (BNL-HCC) and HUVEC were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and KURABO (Osaka, Japan), respectively. BNL-HCC cells are an adherent chemically transformed mouse liver cell line derived from the normal BALB/c embryonic liver cell line, BNL CL2 (American Tissue Culture Collection, Manasses, VA; TIB 73) as described previously (23). The cells were grown in the media recommended by the respective suppliers.

Animal treatment. A total of 40 male 6-week-old BALB/c mice were purchased from Japan SLC, Inc.(Hamamatsu, Shizuoka, Japan). They were housed under controlled temperature conditions and relative humidity, with 10-15 air changes per hour (hr) and light illumination for 12 hr a day.

To create the allograft model, 1×10^6 of BNL-HCC cells were injected into the flank of BALB/c mice. The mice were randomly divided into four groups (n=10 in each group). Group 1 (G1) consisted of untreated mice that served as a control group. Mice in G2 and G3 received 1×10^4 IU of IFN twice a week with subcutaneous injection on the contralateral side of the tumor, and 2 mg/kg/day of PE by daily gavage, respectively. It has been reported that the doses of these agents are almost comparable to those used in the clinical practice (12, 20). The combination treatment of PE and IFN group was designed as G4. The animals were allowed free access to food and water throughout the acclimation and experiment protocols. The tumor volume was measured twice a week, and the mice were killed at 32 days after the tumor cell implantation. The next experiment was conducted to examine the effect of PE and IFN on the fully established tumor growth. In this experiment, either IFN or PE administration was started on day 14 (the mean tumor volume was 200 mm^3). The mice were killed 48 days after the tumor cell implantation. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

Immunohistochemistry. For determination of the *in vivo* angiogenesis, we employed immunohistochemical detection of platelet /

EC adhesion molecule (PECAM /CD31), which is widely used as a marker of neovascularization, in frozen sections of tumors with the same size to avoid the necrotic effect of hypoxia as described previously (24). The immunostained microvessel length was assessed under x 200-fold magnification. In each tumor sample, five areas showing the highest density of staining were selected for counting. In counting, the large vessels with a thick muscular wall or with a lumen greater than 50 μm in diameter were excluded. These immunopositive vessels were evaluated with Adobe Photoshop and NIH image software as described previously (25). Apoptosis was detected with DNA fragmentation products that were stained by *in situ* 3' end labeling (terminal deoxynucleotidyl transferase-mediated dUTP nick labeling [TUNEL] with paraffin-embedded sections. The TUNEL positive cells were counted in the PE-, IFN-treated and control groups, using a light microscope. In each tumor, the positive cells in 10 high-power fields at a magnification of x 400 were examined as described previously (25).

Measurement of VEGF expression in tumors. We measured the VEGF protein expression level in the tumor. Because a different size of tumor may cause different hypoxic conditions, which strongly induced VEGF (26), five tumors having the same size were chosen from each group.

The tumor samples were prepared as described previously (27). After the protein concentration was equalized, the VEGF level was measured with an ELISA kit (R&D Systems, Minneapolis, MN, USA) in accordance with the supplier's instructions.

***In vitro* proliferation and angiogenesis assay.** Because PE is a pro-drug, the active form, perindoprilat, was used for the *in vitro* studies. The *in vitro* proliferation was determined by MTT assay as described elsewhere (27). The cell proliferation was quantified via conversion of tetrazolium, 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by cells cultured in 12-well plates. MTT was added to each well at a final concentration of 5 mg/ml in the presence or absence of perindoprilat (100 μM) and/or IFN (10, 10^2, 10^3 IU/ml). After 4-hr incubation at 37℃ with MTT, the untreated MTT and medium were removed, and 2 ml of dimethyl sulfoxide were added to solubilize the MTT formazan. After gentle agitation for 10 min, the optical density of each well, which is directly proportional to the number of living cells, was measured with a 540-nm filter. The absorbance was read with an ELISA plate reader (n = 6 per group). For the *in vitro* assessment of angiogenesis, we used the EC tubule formation assay as previously described (12). Briefly, Matrigel (Becton Dickinson Labware, Bodford. MA, USA) was placed in 6-well

tissue plates (2 ml /well) and allowed to set at 37℃ for 30 min. Then 1.5×10^5 HUVECs were added to each well and incubated in the presence or absence of perindoprilat (1μM) and/or IFN (10 IU /ml) at 37℃ for 20 hr under a 5% CO_2 atmosphere. Semiquantitation of tubule formation was performed in the same way as for the *in vivo* assay.

Statistical analysis. To assess the statistical significance of inter-group differences in the quantitative data, Bonferroni's multiple comparison test was performed after one-way ANOVA. This was followed by Barlett's test to determine the homology of variance.

RESULTS

Effect of IFN and PE on tumor development. We first examined the effect of clinically comparable doses of IFN and PE on HCC development. As shown in Fig. 1A, single agent treatment with either IFN (G2) or PE (G3) showed a marked inhibitory effect on HCC development as compared to the control group (G1) ($p<0.05$ and 0.01, respectively). The inhibitory effect in G2 was weaker than that in G3 ($p<0.05$). Mice treated with IFN plus PE (G4) showed a significant decrease in the tumor volume as compared those in the control group, G2, and G3. We next examined whether the inhibitory effects of PE and IFN could be found even after the

tumor was established. When the tumor volume reached about 200 mm^3, either PE or IFN treatment was started. In this experiment, IFN alone did not show a significant inhibitory effect. However, IFN significantly augmented the tumoricidial effect of PE, and the combination effect of IFN plus PE was more potent than the additive effect of both compounds. The single-agent treatment and even the combination treatment at the current doses did not affect the health status, such as body weights during the experiment (data not shown).

Tumor neovascularization and apoptosis. To determine whether the combined inhibitory effect of IFN and PE on the tumor development was accompanied by suppression of neovascularization, we examined the tumor expression level of CD31. As shown in Fig. 2, and similar to the result of HCC development suppression, the CD31-positive vessels in the tumors of either G2 or G3 were significantly fewer than those in the control group. The combination treatment of IFN and PE mostly attenuated the CD31-positive vessels in the tumor. To perform semi-quantification of the neovascularization in the tumor, we employed computer-assisted image analysis techniques as described previously (12). A semi-quantitative analysis of the CD31-positive vessels in G2 or G3 revealed a statistically significant suppression as compared to the control

group ($p<0.05$ and $p<0.01$, respectively). The combination treatment of IFN and PE exerted a much stronger inhibition of CD31-positive vessels in the tumor as compared to G3 ($p<0.05$) (Fig. 3). On the contrary, the number of TUNEL-positive cells was significantly increased by treatment with IFN or PE ($p< 0.05$ and $p<0.01$, respectively). Moreover, the combination treatment of IFN and PE revealed much more TUNEL-positive cells in the tumor than in G3 ($p<0.05$) (Figs. 4 and 5). The incidence of apoptosis in the tumor almost corresponded to the effect of tumor development inhibition. Histological examination of H&E-stained sections did not exhibit a numerical increase of the inflammatory cells, mainly macrophages, and extensive necrosis in G2 and G4, suggesting that suppressive effect of IFN was not due to the immunoresponse alteration at the currently used low dose (data not shown).

VEGF expression in the tumor. Since we previously observed that PE treatment suppressed the VEGF expression in the experimental HCC model (12), we also examined the combined effect of IFN and PE on the VEGF expression of the tumor in the current study. As shown in Fig. 6, the VEGF expression in the tumor was significantly suppressed by treatment with IFN or PE ($p<0.05$). The combination treatment with IFN and PE exerted a much stronger inhibition of VEGF expression in the

tumor than in G3 ($p<0.05$). We also examined the mRNA expression of VEGF in the tumor by RT-PCR, and we found a similar inhibitory effect of IFN and PE (data not shown).

Effect of IFN and PE on HCC and EC proliferation *in vitro*. To elucidate the possible mechanism of the inhibitory effect of IFN and PE, we examined whether the inhibitory effects of IFN and PE were related to cytotoxicity. As shown in Fig. 7A, we found that neither IFN ($1x10^3$ IU/ml) nor PE (100 μM) influenced the *in vitro* proliferation of HCC tumor cells *in vitro*. Even the combination treatment of IFN and PE did not show any inhibition of the *in vitro* HCC cell proliferation. At a dose of 100 μM, PE did not show any inhibitory effect on EC, either. On the other hand, IFN revealed a marked inhibitory effect on EC proliferation even at a low dose (10 IU/ml) ($p<0.01$) as compared to the untreated control group. IFN treatment exerted a marked inhibition of EC proliferation in a dose-dependent manner (Fig. 7B). PE did not show any additional inhibitory effect to that of IFN on EC proliferation (data not shown).

Effect of IFN and PE on *in vitro* angiogenesis. We also investigated the *in vitro* EC tubule formation in the presence or absence of IFN and PE. We found that IFN or PE alone significantly inhibited the EC tubule formation in Matrigel, and that the combination treatment of IFN

and PE almost completely attenuated the EC tubule formation at low doses (10 IU/ml and 1 μM, respectively) (Fig. 8). The inhibitory effect of PE seemed to be stronger than that of IFN. Our semi-quantitaitve analysis showed that the total length of tubules formed in the IFN- or PE-treated cultures were significantly less than in the untreated control culture ($p<0.05$ and $p<0.01$, respectively). The combination treatment of PE and IFN resulted in a further inhibition of EC tubular formation than PE alone ($p<0.05$) (Fig. 9).

DISCUSSION

Hepatocellular carcinoma is the most frequent primary malignancy of the liver, and its incidence appears to be rising in the United States and other developed countries, although the United States and Western Europe still have a low incidence of HCC (28, 29). The prognosis of HCC is still poor since most cases are found in conjunction with chronic liver diseases, such as liver cirrhosis. Radical operation is the only curative modality for HCC, but it is only appropriate in the minority of patients due to the limited hepatic reserves. Therefore, various palliative therapeutic modalities, such as trans-arterial embolization (TAE), percutaneous intratumoral ethanol injection (PEIT) and orthotopic transplantation, have been employed, but

no satisfactory treatment for HCC has been available yet (28, 29). A novel approach is required if the overall survival rate of patients with HCC is to be significantly improved.

It is now widely recognized that any solid tumor can not grow even beyond a few mm in size without angiogenesis (1, 2). One of the characteristic features of HCC in the clinical practice is hypervascularity. As expected, it has been shown that several angiogenic factors, such as VEGF, are significantly up-regulated in the human HCC tissues than in the surrounding non-cancerous lesions, and angiogenesis plays a pivotal role in the experimental HCC development (26, 30, 31). Accordingly, it is likely that anti-angiogenic therapy would be a promising approach against HCC. In this study, we found that IFN or PE exerted a significantly inhibitory effect of HCC development associated with suppression of angiogenesis, and the combination treatment of IFN and PE showed a more inhibitory effect than the single-agent treatment at clinically comparable low doses. These inhibitory effects were also detected even after the tumor was fully established.

The pivotal role of VEGF in tumor angiogenesis has been demonstrated in various experimental systems including HCC (26, 32, 33). Inhibition of VEGF function, *e.g*., by neutralizing the monoclonal antibodies to VEGF

or VEGFR-2, by antisense gene or ribozyme transfer, or by specific inhibitors of VEGF signaling cascade, has unequivocally demonstrated the requirement of VEGF for tumor angiogenesis and consequently tumor development (26, 33). In HCC, we previously reported that VEGF tightly regulated HCC development, and that suppression of the VEGF-VEGFR interaction or VEGF-mediated signaling pathway significantly attenuated the HCC development and angiogenesis (23, 24, 32). The VEGF gene expression is regulated several factors, including AT-II (26, 34). AT-II is a product of the renin-angiotensin system, which has been reportedly activated in patients with chronic liver diseases, such as cirrhosis (35, 36). We have shown previously that inhibition of AT-II by PE significantly suppressed the VEGF expression in the tumor (12, 37). It has been reported that IFN can also down-regulate several angiogenic factors, such as bFGF, IL-8, MMP-2, MMP-9, and VEGF (38-41). We observed in the current study that IFN and PE treatment markedly suppressed the VEGF expression in HCC. These results suggest that the inhibitory effect of IFN and PE was at least partly mediated by suppression of VEGF in HCC.

It has been shown that treatment with anti-angiogenic agents induced a marked increase of apoptosis in the tumors, whereas it did not alter the tumor cell proliferation itself (1, 6, 25). In this study, immunohistochemical

analysis by TUNEL assay revealed that IFN and PE significantly increased apoptosis in the tumor. On the contrary, the tumor cell proliferation was not altered by treatment with IFN and PE (data not shown). It is an important point to determine whether apoptosis was observed mainly in the EC or in the tumor cells. We do not have an exact answer at this time. Although we performed a double immunohistochemical analysis with CD31 and TUNEL in a couple of times, we failed to obtain a good result. The background was very intense, and the interpretation was very difficult (data not shown). In the current study, we found that IFN significantly inhibited the EC proliferation *in vitro*, whereas neither IFN nor PE affected the HCC cell proliferation. It has been reported that IFN-α and IFN-β exerted different activities on EC proliferation. IFN-β exerted 100-1000-fold stronger inhibitory effect on EC proliferation than IFN-α (42). Both IFN and PE exerted a significant inhibitory effect on EC tubular formation *in vitro*. Furthermore, IFN and PE attenuated the expression of VEGF in the tumor, which was known as a survival factor for the EC. These findings, taken together, suggest that IFN and PE first induced the EC apoptosis, and this might induce the secondary apoptosis of the tumor cells.

The use of anti-angiogenic agents as monotherapy in treating patients with advanced cancer has not yet shown a significant efficacy (1, 6). The

limitations of anti-angiogenic monotherapy in this setting were in fact predicted by preclinical studies with the angiogenesis inhibitors endostatin and angiostatin. It has been reported that the combination treatment of anti-angiogenic agents, such as endostatin and angiostatin, revealed a synergistic inhibitory effect on the tumor development and angiogenesis (6, 43). It has been also reported that the combination of TNP-470, which is one of the anti-angiogenic agents under clinical trials, and IFN inhibited angiogenesis synergistically (44). We employed IFN and PE in the current study, and we found the combination treatment of these agents exerted a significant tumor inhibitory effect associated with suppression of angiogenesis. PE is widely used currently without serious side effects in more than 100 countries, and the safety of administration to the patients with liver cirrhosis has been reported (45, 46). Also it has been shown that the long-term treatment with low dose of IFN is tolerable to the patients with chronic liver disease (21, 22). A noteworthy finding in this study was that the combined inhibitory effect of IFN and PE could be observed at clinically comparable low doses of both agents as described previously (12, 20). PE had no effect on EC proliferation whereas it significantly suppressed the EC tubular formation *in vitro*. On the other hand, IFN exerted a potent inhibitory effect on EC proliferation although the

inhibitory effect of EC tubular formation was not strong. Furthermore, we previously found that PE exerted a marked inhibition of VEGF in the tumor cells both *in vitro* and *in vivo* (12), and that PE also inhibited the VEGF-induced EC migration (37). It would be possible that the co-ordination of these different biological activities resulted in the *in vivo* combination tumoricidial effect of PE and IFN. Further studies are required to elucidate the exact mechanism in the future.

In summary, we have shown here that the combination treatment of ACE inhibitor, PE, and IFN significantly inhibited HCC development and angiogenesis along with suppression of VEGF. Since both agents are currently widely used in the clinical practice, this combination regimen may represent a potential new strategy for HCC therapy in the future.

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FIGURE LEGENDS

Figure 1. Effects of IFN and PE on BNL-HCC development. Group1 (G1) is the untreated control group (open circle). Animals in G2 (closed circle) and G3 (open square) received IFN and PE at doses of $1x10^4$ IU/twice a week and 2 mg/kg/day, respectively. The combination treatment of IFN and PE was designed as G4 (closed triangle). IFN and PE were administered from the beginning of the experiment **(A)** and from day 14 (the mean tumor volume was 200 mm^3)**(B)**, respectively. The tumor volume was determined by calipers at the indicated time points. Each point represents the mean ± SD (n= 10). * and ** indicate statistically significant differences between the indicated experimental groups ($p< 0.05$ and 0.01, respectively). The black arrow indicates the time point at which PE and IFN started (day 14).

Figure 2. Immunohistochemical analysis of CD31 expression in the tumor. Tumor vascularization was visualized by immunostaining of the CD31 vascular endothelial adhesion protein. **(A)(B):** The control untreated group. **(C), (D):** IFN ($1x10^4$ IU)-treated group. **(E)(F):** PE (2 mg/kg/day)-treated group. **(G)(H):** IFN and PE combination treated group.

The original magnifications of **(A)(C)(E)(G)** and **(B)(D)(F)(H)** were x 40 and x 200, respectively.

Figure 3. Semi-quantitative analysis of CD31-immunopositive vessels. The length of CD31-positive vessels in the tumor was measured by an image analysis system as described in the “Materials and Methods” section. The data represent the mean ± SD (n=5). * and ** indicate statistically significant difference between the indicated experimental groups ($p< 0.05$ and 0.01, respectively). Cont: The untreated control group. IFN, PE: IFN ($1x10^4$ IU/twice a week)- and PE (2 mg/kg/day)-treated group, respectively. IFN+PE: IFN and PE combination treated group.

Figure 4. Immunohistochemical analysis of apoptosis in the tumor. The apoptotic cells were visualized by immunostaining with TUNEL assay. **(A):** The control untreated group. **(B), (C):** IFN ($1x10^4$ IU/twice a week)- and PE (2 mg/kg/day)-treated group, respectively. **(D):** IFN and PE combination treated group. The arrow indicates the apoptotic cell in the tumor. The original magnifications were x200. Scale bar: 100 μm.

Figure 5. Semi-quantitative analysis of TUNEL-immunopositive

apoptotic cells. The data represent the mean ± SD (n=5). * and ** indicate statistically significant difference between the indicated experimental groups ($p< 0.05$ and 0.01, respectively). Cont: The untreated control group. IFN, PE: IFN ($1x10^4$ IU/twice a week)- and PE (2 mg/kg/day)-treated group, respectively. IFN+PE: IFN and PE combination treated group.

Figure 6. Effects of IFN and PE on the VEGF expression in the tumor. The VEGF protein level was measured by ELISA as described in the "Materials and Methods" section. The data represent the mean ± SD (n=5). * indicates statistically significant difference as compared to the control group ($p< 0.05$). Cont: The untreated control group. IFN, PE: IFN ($1x10^4$ IU/twice a week)- and PE (2 mg/kg/day)-treated group, respectively. IFN+PE: IFN and PE combination treated group.

Figure 7. Effects of IFN and PE on the HCC cells **(A)** and EC **(B)** proliferation *in vitro*. Cell proliferation was measured by MTT assay after harvest from day 1 to day 5 as described in the "Materials and Methods" section. Each blot represents the mean ± SD (n=5). * and ** indicate statistically significant difference between the indicated experimental groups ($p< 0.05$ and 0.01, respectively). Cont: The untreated control group

(open circle). PE: perindoprilat (the active form of perindopril: 100 μM)-treated group (closed circle). IFN (10, 10^2, 10^3): IFN-treated groups at doses of 10, 10^2, 10^3 IU/ml (closed square, closed triangle, and open triangle, respectively). IFN+PE: IFN (10^3 IU/ml) and perindoprilat (100 μM) combination treated group (open square).

Figure 8. Effects of IFN and PE on the *in vitro* EC tubular formation on Matrigel. **(A):** Control untreated group. **(B), (C):** IFN (10 IU/ml)- and PE (perindoprilat; the active form of perindopril: 1 μM)-treated group, respectively. **(D):** IFN and PE combination treated group.

Figure 9. Semi-quantitative analysis of EC tubular formation on Matrigel. The total tubule length was measured by an image analysis system as described in the “Materials and Methods” section. The data represent the mean ± SD (n=5). * and ** indicate statistically significant difference between the indicated experimental groups ($p< 0.05$ and 0.01, respectively). Cont: The untreated control group. IFN, PE: IFN (10 IU/mL)- and PE (perindoprilat: 1 μM)-treated group, respectively. IFN+PE: IFN and PE combination treated group.

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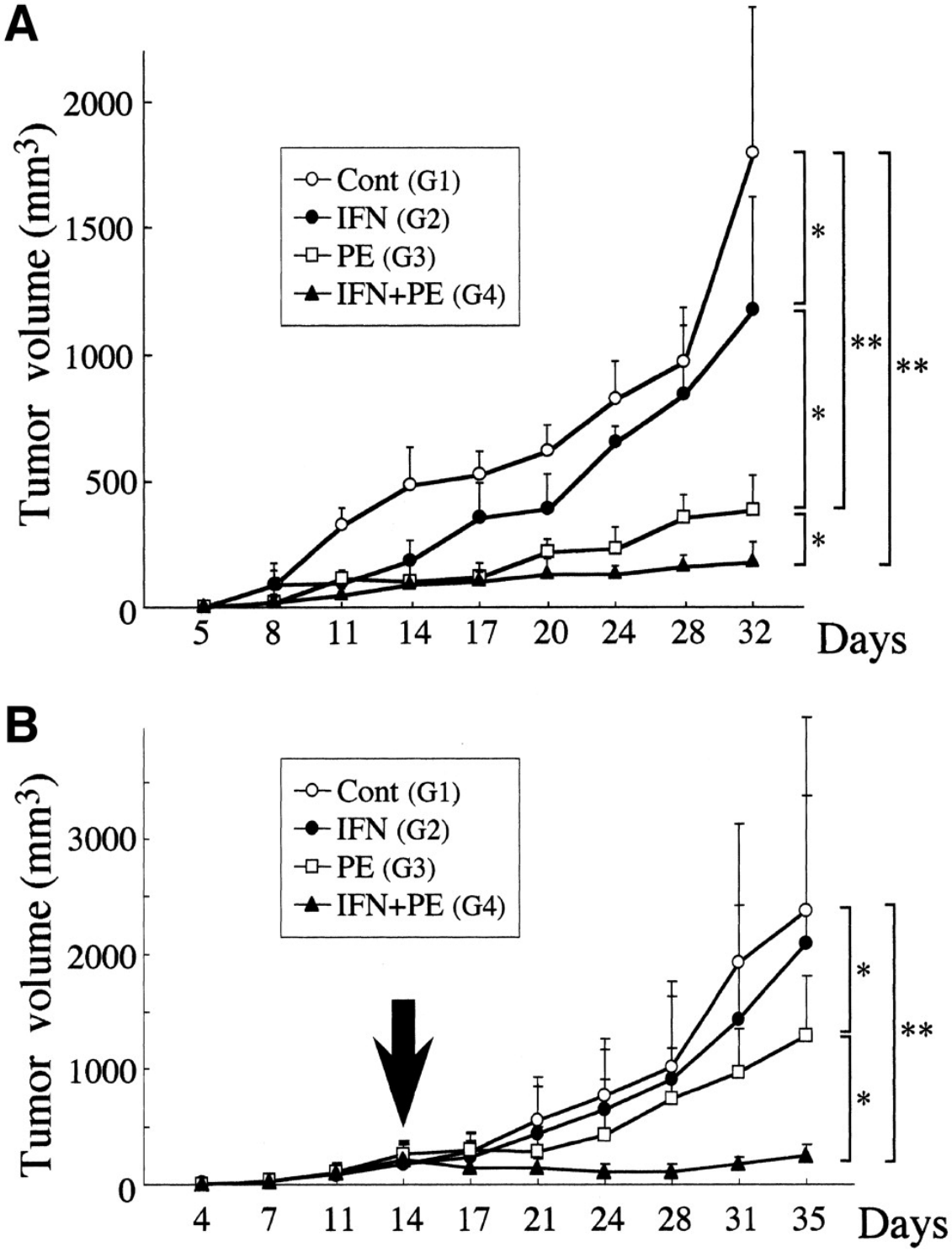
Fig.1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IFN (G2)
PE (G3)
IFN+PE (G4)
*
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*
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5
8
11
14
17
20
24
28
32
Days
B
Tumor volume (mm3)
3000
2000
1000
0
Cont (G1)
IFN (G2)
PE (G3)
IFN+PE (G4)
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4
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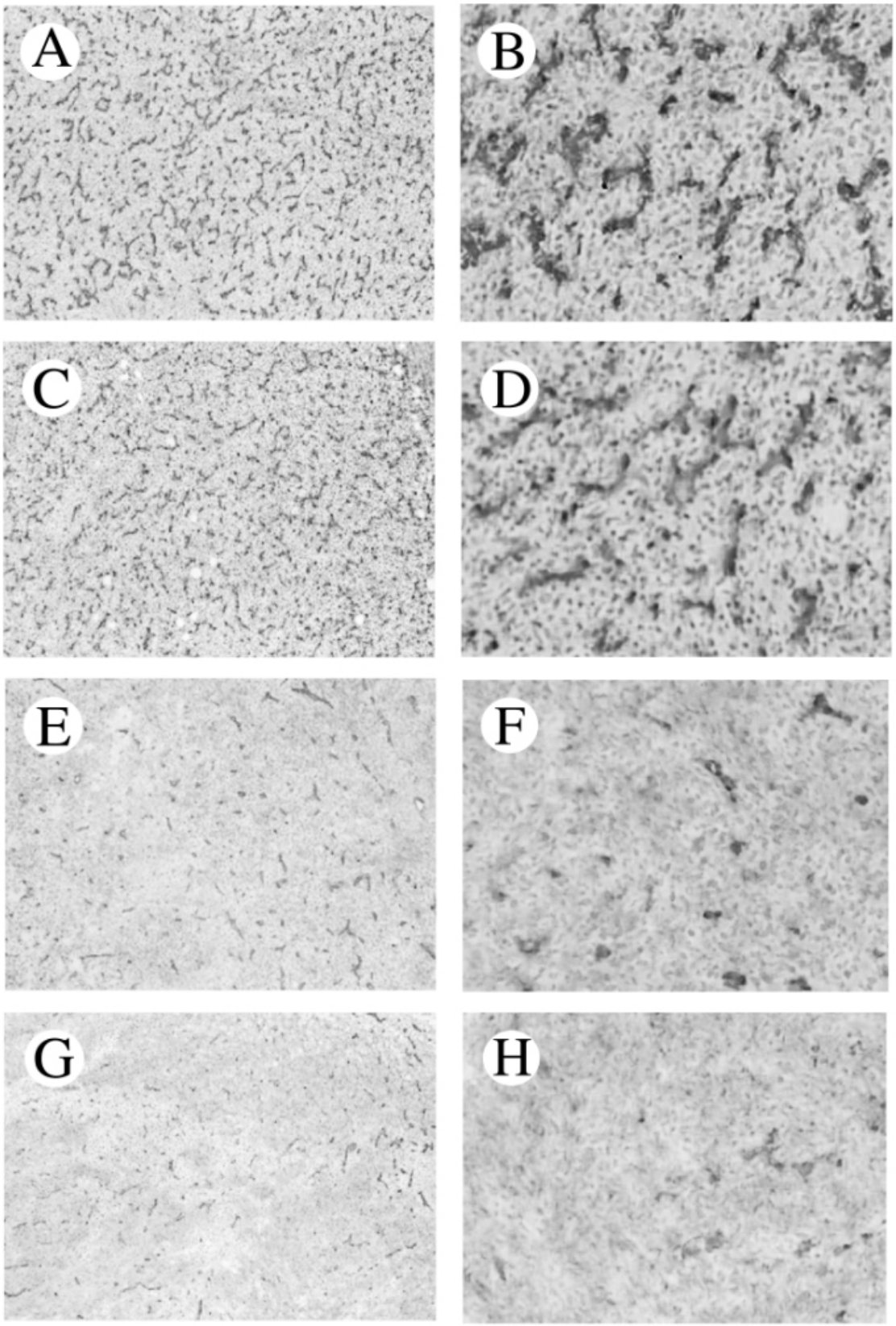
31
35
Days

Fig.2



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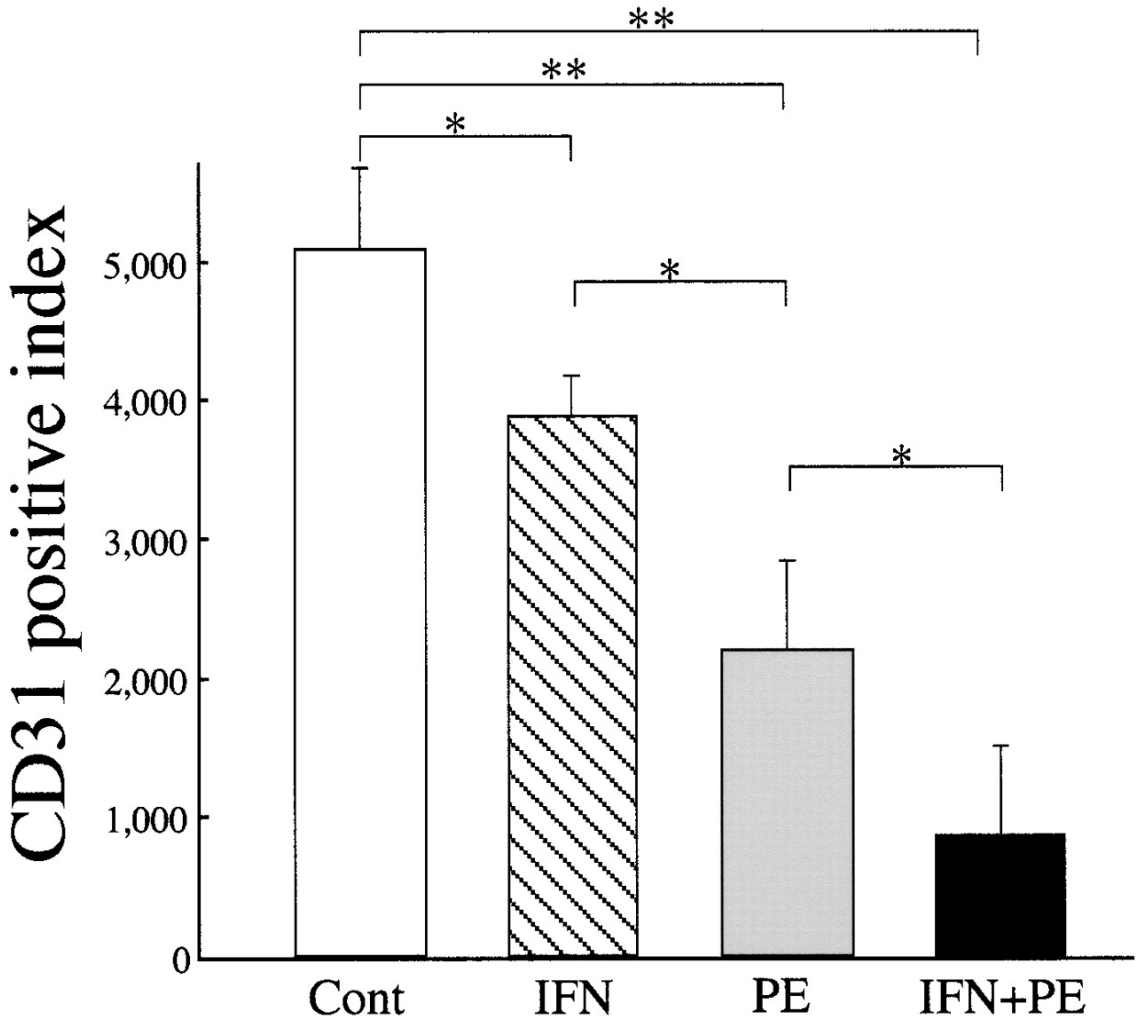
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IFN
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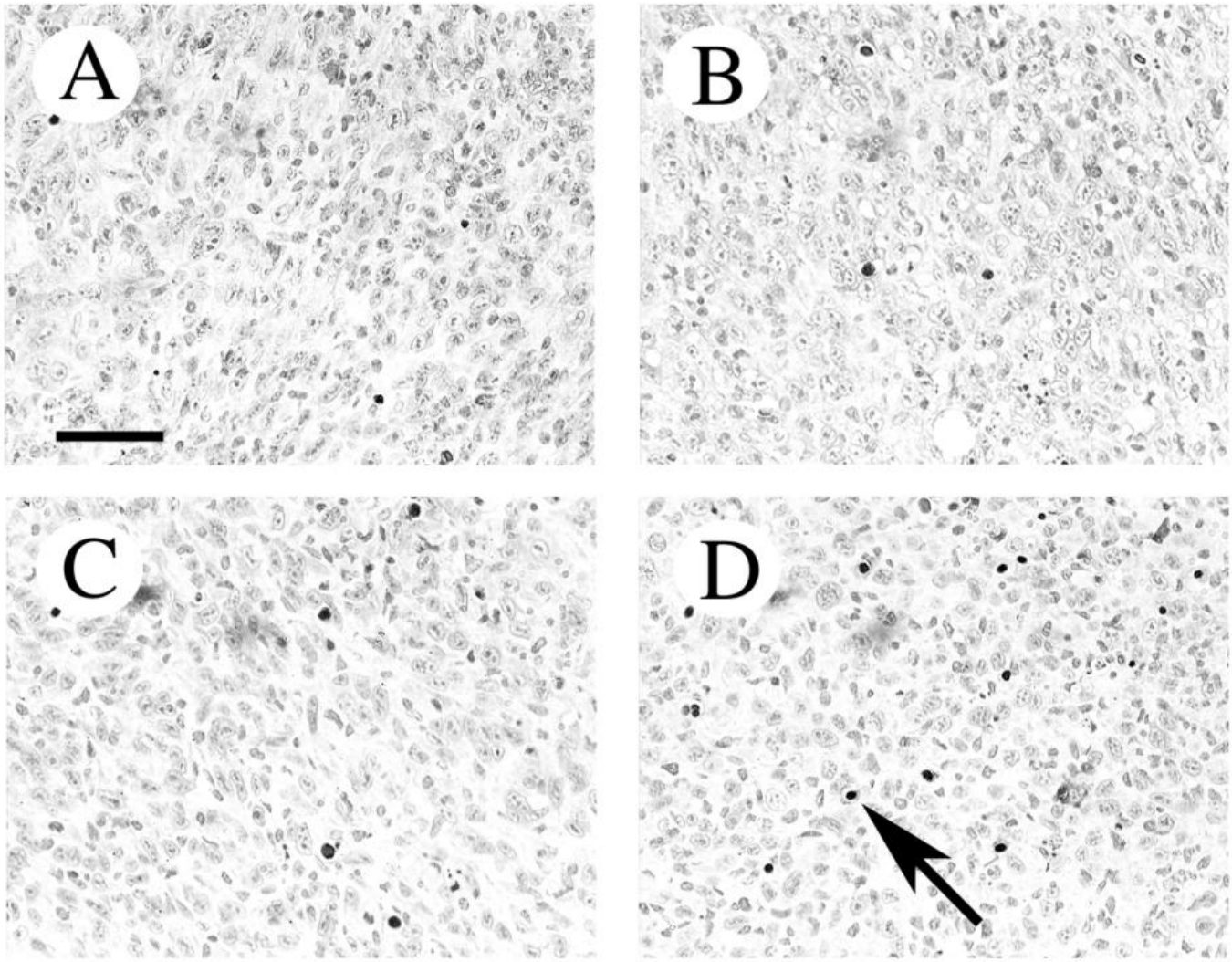
Fig.4



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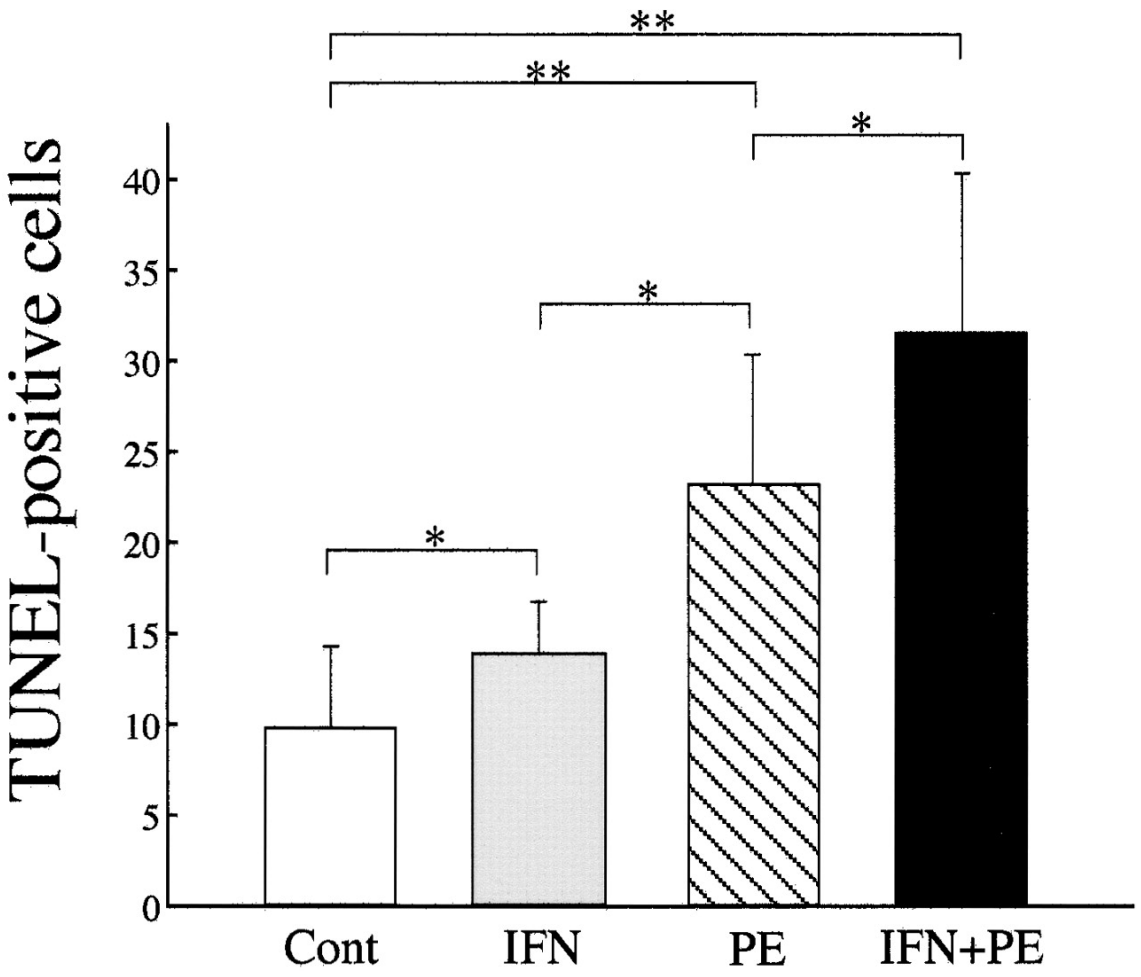

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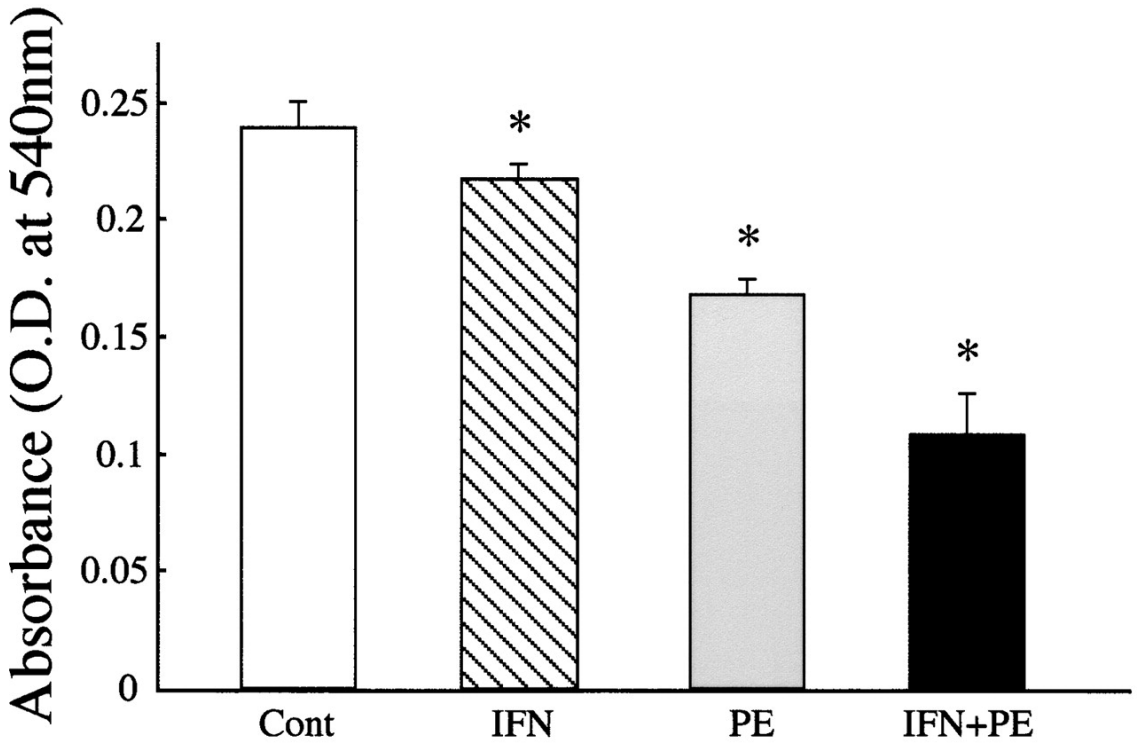

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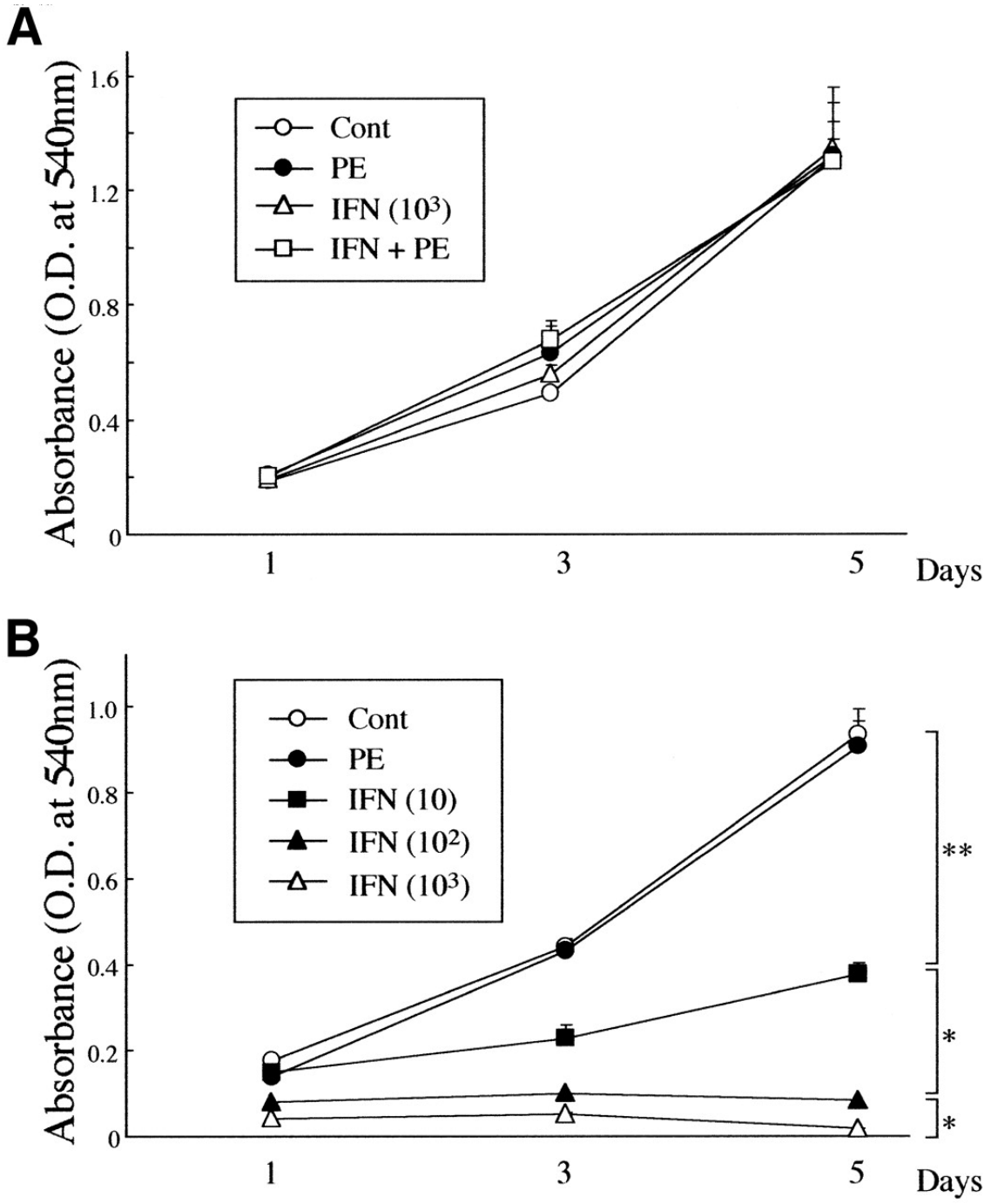
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0.8
0.4
0
Cont
PE
IFN (10³)
IFN + PE
1
3
5
Days
B
Absorbance (O.D. at 540nm)
1.0
0.8
0.6
0.4
0.2
0
Cont
PE
IFN (10)
IFN (10²)
IFN (10³)
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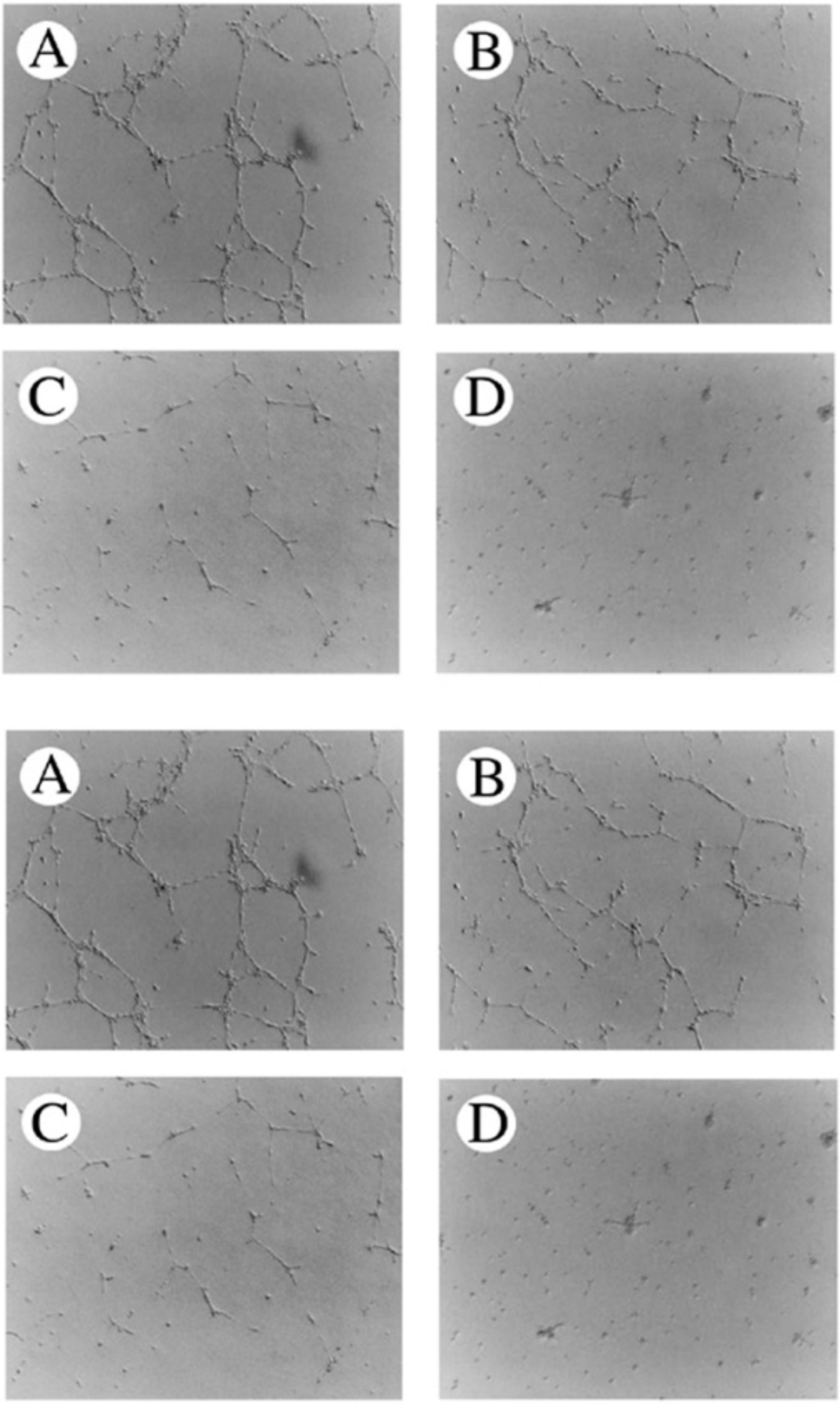
*
1
3
5
Days

Fig.8



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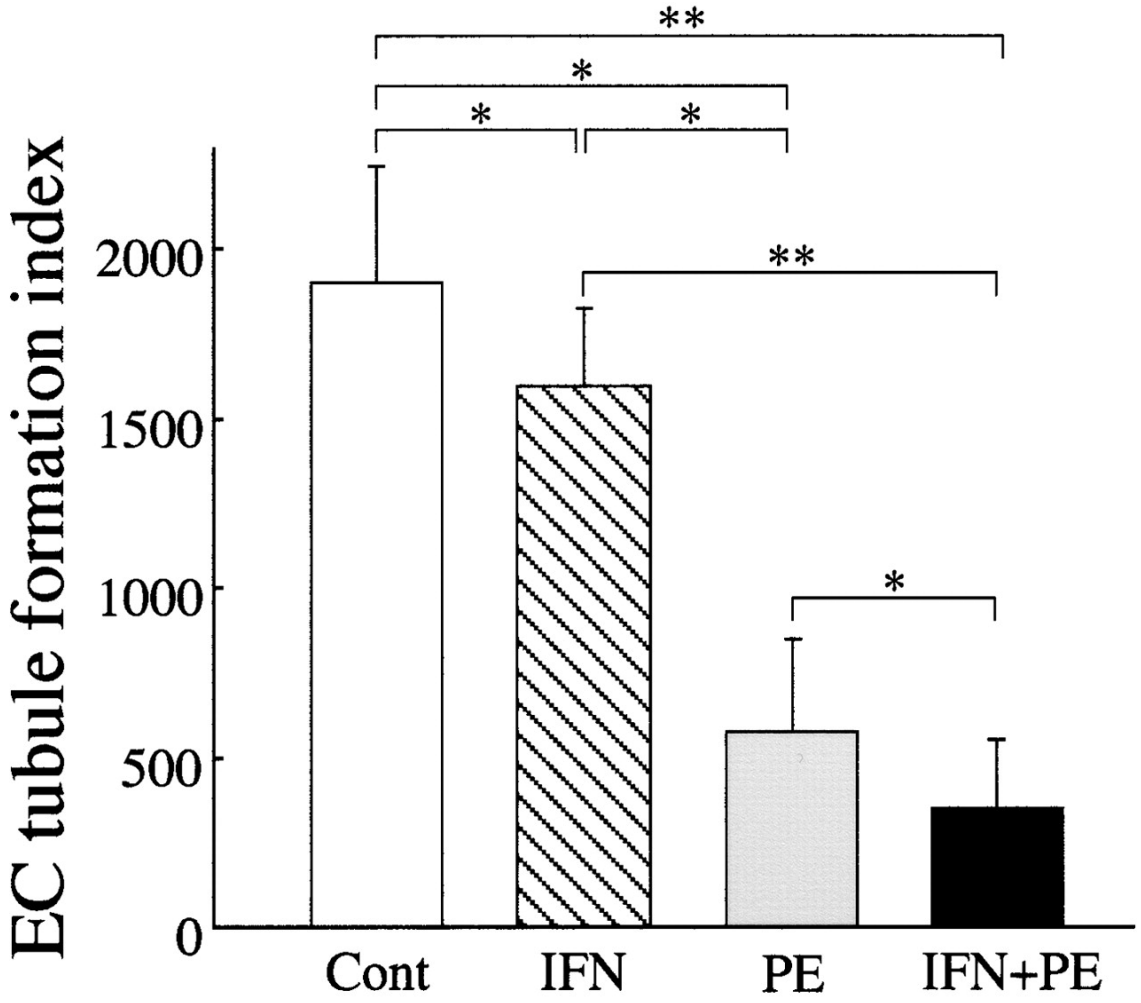
EC tubule formation index
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