学 位 論 文

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Synergistic antitumor effects of human interferon-β and interferon-γ on human gastric adenocarcinoma cells

Seiichi NAGAO[1,2], Akinobu NAKANO[1], Kouzo MORITAKE[2], Shigeru MORIKAWA[1] and Takayuki HARADA[1]

Departments of [1]Pathology and [2]Neurosurgery,

Shimane Medical University,

89-1 Enyacho, Izumo 693, Japan

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SUMMARY

The combined effects of different classes of interferon (IFN) on human gastric adenocarcinoma cell line HPE-GAC-2 (GAC-2) were investigated *in vitro*. Synergistic effects of IFN-β and -γ, causing cell death, were observed in a dose-dependent manner. IFN-β modulated the sensitivity of GAC-2 cells to the cytocidal effect of IFN-γ. The modulatory effect was independent of the cytostatic or cytocidal activity of IFN-β since brief contact with IFN-β did not influence the growth kinetics of the cells and was effective in modulate the cellular sensitivity to IFN-γ. A brief pretreatment of the cells with IFN-α or -β resulted in a variable level of modulation, whereas coculture of the cells with IFN-β or -α and IFN-γ had a similar cytotoxic effect indicating a different level of activity induced by IFN-α and -β with the common type I receptor. These results suggest that IFN-β-induced modulation was receptor-mediated. Phase contrast microscopy showed evidence of apoptotic cell death induced by one or more IFN agents. The morphological changes included chromatin condensation and nuclear fragmentation which were apparent at as early as 6 hr of culture with a high concentration of IFN(s). Our results indicate that IFN-β and IFN-α enhance the *in vitro* apoptotic effects of IFN-γ against GAC-2 tumor cells.

Key words: apoptosis, interferon, antitumor activity, cancer, IFN-β, IFN-α, IFN-γ

Interferons (IFN) are a family of glycoproteins with antiviral, antiproliferative and immunomodulating properties. The three types of IFN, which can be distinguished by their acid stability, primary sequence, and chromosomal location and organization, act on a variety of target cells via specific class I (IFN-α and -β) and II (IFN-γ) receptors. A large number of the biochemical actions of IFN can be attributed to gene activation and stimulation of the synthesis of several proteins of known and unknown function. However, the mechanisms underlying other complex biological and cellular changes triggered by IFNs are poorly understood.

IFNs are used clinically as anticancer agents against selected malignancies, such as AIDS-related Kaposi sarcoma [1], renal cell carcinoma [2], certain lymphomas [3], chronic myelogenous leukemia [4], hairy cell leukemia [5], multiple myeloma [6] or glioma [7]. The antitumor effects of IFN *in vivo* can be ascribed to both direct action on tumor cells and indirect effect through the modulation of the host immune system [8], but the general response to IFN is not always as good as the response to cytotoxic chemical drugs [9, 10]. In contrast to the limited usage in cancer patients, accumulating evidence indicates that a variety of cancer cells are sensitive to IFNs *in vitro*. The discrepancy between *in vivo* and *in vitro* anti-cancer effects is partly due to the heterogeneous response of cancer cells to IFNs. Because IFNs show relatively weak cytotoxic effects but can modulate cellular activities, e.g., inhibition of cell cycle progression [11], it has been postulated that they may be best used as biological response modifiers, in combination with other cytotoxic agents [12].

In the present study, we showed profound cytotoxic effects and modulation of sensitivity by different classes of IFN *in vitro* to a human gastric adenocarcinoma cell

line. IFN-γ exhibited cytotoxic activity on these cells, and coculture with IFN-β showed a synergistic cytocidal effect. This synergism was produced not by the cytostatic or cytocidal activity of IFN-β but by modulating the sensitivity of the cells to IFN-γ. Furthermore, we also showed that IFN-induced cell death was that of apoptosis.

MATERIALS AND METHODS

Cell lines and cultures

A gastric adenocarcinoma cell line, HPE-GAC-2 (GAC-2), which was established by Morikawa et al. [13, 14] and has been maintained *in vitro* in our laboratory, was used in the present study. Cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal calf serum (RPMI-FCS) with kanamycin (0.06 mg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in 95% air. Logarithmic growth of the cells and viability exceeding 95% were maintained by subculturing twice every week.

Interferons and anti-IFN-β antibody

Human fibroblast IFN-β and recombinant IFN-γ were provided by Toray Co., and natural IFN-α was provided by Sumitomo Pharmaceutical Co.. The specific activity was 1×10^7 IU or U/mg of protein. The glycoprotein compounds were dissolved in saline in the present experiments and the stock solution was stored at -70°C (IFN-β and -γ) or 4°C (IFN-α) until use. A working solution of IFN in RPMI-FCS was prepared just before the experiment. The stock solution of anti-IFN-β monoclonal antibody(mAb), a gift from Toray Co., was first diluted (1:4000) before use. One milliliter of the diluted

anti-IFN-β mAb neutralized 90% of 10 IU of IFN-β. IFN-β and anti-IFN-β mAb were preincubated at room temperature for 30 min in a 24-well (Falcon #3047) plate before commencement of cell culture.

Assay for antiproliferative activity of interferons

Duplicate cultures were prepared using a cell concentration of 1×10^5 cells/ml/well in a 24-well plate with varying concentrations of IFNs. The number of cells and their viability at any time interval were assessed by counting the cells in a hemocytometer using trypan blue dye. All data described herein are representative of at least three independent experiments with similar results.

For pretreatment of HPE-GAC-2 cells, IFN-α or -β in a one-tenth the volume of cell suspension was added at room temperature to 5×10^5 cells/ml cell suspension in a conical centrifuge tube (Sumibei #MS-56500). Immediately after the addition of IFN, the cells were centrifuged three times at 150 *g* for 10 min to remove the agent, and cultured with varying concentrations of IFN-γ in a 24-well plate, as described above. In some experiments, IFN-γ was added after commencement of the culture.

Analysis of synergy of IFN-β and IFN-γ

We analyzed the combined effects of IFN-β and IFN-γ using the improved isobologram method [15, 16]. Based on the individual dose-response curves of IFN-β and IFN-γ, three isoeffect curves were prepared as follows. Mode I line: When a dose of IFN-γ is selected, there remains an increment in isoeffect to be produced by IFN-β. If

the two agents act independently, the addition is performed by taking the increment in doses, starting at zero, that are added up to the determined inhibitory concentration (IC_{50} in this experiment). Mode II (IFN-γ) line: When the dose of IFN-γ is chosen, as isoeffect curve can also be calculated by taking the dose increment of IFN-β that produces the required contribution to the total effect up to IC_{50}. Mode II (IFN-β) line: Similarly, when the dose of IFN-β is chosen, an isoeffect curve can be calculated by selecting the dose increment of IFN-γ that produces the required contribution to IC_{50}. The experimental data points pertaining to the combination of IFN-β and IFN-γ were plotted. When the data points are within an area enclosed by the three isoeffect curves (envelope of additivity), the effect is considered additive. Otherwise, when the data points fall to the left of the envelope, the effect is considered synergistic.

Cellular morphology

Cellular morphology was observed by phase contrast microscopy during culture, and cytospin preparations were stained with May Gruenwald and Giemsa solutions.

Statistical analysis

Data were expressed as the mean ± SD. Differences between the effects of two compounds were examined by the Student *t*-test. A p value less than 0.05 denoted the presence of significant statistical differences.

RESULTS

Sensitivity of human gastric adenocarcinoma cells to human IFN(s) in vitro.

Preliminary experiments showed that human gastric adenocarcinoma cell line HPE-GAC-2 was sensitive to different classes of IFNs. The cell line was therefore selected for further experimentation. IFN-β exhibited mild cytostatic and cytocidal effects against GAC-2 at relatively higher concentrations (Fig. 1A and B). In contrast, IFN-γ produced a strong cytocidal effect and caused total cell death at 96 hr of culture in a dose-dependent fashion (Fig. 1C and D). We next examined whether culture in the presence of both drugs could potentiate the antitumor effect. These tests showed a more profound cytotoxicity even at low concentrations of IFN-γ (3 or 30 U/ml) when combined with 30 or 300 IU/ml of IFN-β (Fig. 2A). A synergistic effect was evident by isobologram analysis within 24 hr of culture (Fig. 2B). The combined effects of IFN-γ and IFN-β were completely abolished by preincubation of IFN-β with anti-IFN-β mAb prior to the start of culture (Fig. 3) suggesting that the antibody neutralized IFN-β and blocked its interaction with specific receptors on the GAC-2 cells.

Effects of pretreatment of GAC-2 cells with IFN-β on subsequent culture with IFN-γ

IFN-β is known to modulate cellular sensitivity to various agents. Thus, in the next series of experiments we examined in more detail the role of IFN-β in enhancing the sensitivity of GAC-2 cells to IFN-γ. Preculture of GAC-2 cells for 24 hr with a low dose of IFN-β (3 IU/ml) enhanced the sensitivity of these cells to IFN-γ (data not shown). In the next step, we examined the time required for pretreatment of cells with

IFN-β to modulate the action of IFN-γ. Different amounts of IFN-β were added to the cell suspension, but the cells were washed immediately afterwards as described in Materials and Methods. As shown in Figure 4A, IFN-β alone had little or no effect on cell viability, suggesting no substantial amount of IFN-β was carried over into the subsequent culture. This short contact with IFN-β, however, markedly enhanced the sensitivity of GAC-2 cells to the cytotoxic effect of IFN-γ in a dose-dependent manner (Fig. 4B). For example, the dose of IFN-γ necessary to kill 50% of the cells was $3x10^4$ U/ml under control conditions, but diminished to as low as 3 U/ml when the cells were pretreated briefly with $3x10^4$ IU/ml of IFN-β. Moreover, complete cell death was evident at 48 hr in these cultures (data not shown). We further examined whether enhanced sensitivity was observed at very low doses of IFN-γ. A dose-dependent response to very low doses of IFN-γ was clearly observed at 24 hr of culture (Fig. 4C).

In the next set of experiments, we examined the kinetics of IFN-β pretreatment. As shown in Figure 5, 3 U/ml of IFN-γ was added at 0, 24 or 48 hrs to the cultures of GAC-2 cells pretreated with $3x10^4$ U/ml IFN-β. As shown earlier, a marked IFN-γ cytocidal effect was evident in IFN-β pretreated cells, while only a slight growth suppression was observed in the control cells (media alone). However, these experiments clearly showed that it was only when IFN-γ was added at the start of culture, but not 24 or 48 hr later, that it exhibited its cytotoxic effect. Thus, the modulatory effect of IFN-β on GAC-2 cells was brief in the absence of IFN-γ. It was also clear that a residual effect of pretreatment with IFN-β was present even after two cycles of cell growth, i.e., at 48 hr, since suppression of growth caused by adding IFN-

γ to the culture 24 or 48 hr after pretreatment was more evident than that when it was added to control cells (Fig. 5).

Since IFN-α and IFN-β interact with a common type I IFN receptor, we examined whether IFN-α exhibits on GAC-2 cells an effect similar to that of IFN-β. As shown in Figure 6A, the effect of IFN-α was identical to that of IFN-β at every dose examined, irrespective of the presence of IFN-γ in the culture. In contrast to these results, pretreatment with IFN-α had a less striking effect than IFN-β in potentiating the cytocidal effect of low dose IFN-γ at 48 hr of culture (Fig. 6B).

Effect of IFN on cell morphology

Dead cells obtained after treatment with one or more IFNs were readily recognized under phase contrast microscopy based on the characteristic shaggy appearance of the nucleus and reduced light refraction. These features were in sharp contrast to those of viable cells which were characterized by a bright round cellular contour (Fig. 7A). Examination of May Gruenwald- and Giemsa-stained cells showed that the morphological features of the nuclei of dead cells were characteristically those of apoptotic cells. A small number of cells showed condensed chromatin located in the periphery of the nucleus and several cells showed nuclear fragments of various sizes distributed throughout the cytoplasm (Fig. 7B and C). Only a few cells showed blebbing of the plasma membrane after 24 hr treatment with IFN. However, leakage of the fragmented nucleus and attachment of the nuclear material to the outer surface of the plasma membrane were also noted in other cells (Fig. 7C). This phenomenon may have

occurred during the preparation of cytospin specimens and further indicates disintegration of the plasma membrane of apoptotic cells.

DISCUSSION

The major findings of the present study were the enhancement of the sensitivity of HPE-GAC-2, a human gastric adenocarcinoma cell line, to the cytocidal effect of IFN-γ by pretreatment with IFN-β or -α. We also showed that IFN caused apoptosis of these cells.

Previous studies examined the *in vitro* antitumor effects of IFNs using various cancer cell lines. The combination of IFN with chemotherapeutic agents, such as cisplatin, adriamycin or 5-fluorodeoxyuridine [17, 18] or with cytokines, such as TNF [19, 20] or other types of IFN, enhanced the effects of these drugs. Our initial results showed that IFNs were potentially lethal to GAC-2 cells (Fig. 1), and that IFN-β or -γ alone was cytostatic and cytocidal, and cytocidal against these cells, respectively (Fig. 1). Furthermore, pretreatment of these cells with IFN-β sensitized them to IFN-γ (Fig. 4B). This was achieved through either a short period of contact with higher doses (Fig. 4B) or by 24 hr preculture with a very low dose (3 IU/ml) of IFN-β (data not shown). It is noteworthy that the enhanced sensitivity to IFN-γ was produced independent of the cytostatic effect of IFN-β (Fig. 1A and 4A). Since the modulating effect of pretreatment (Fig. 5) and the antitumor effect of IFN-β [21] (our unpublished observations) are reversible, these results do not exclude the possible association of the two types of IFN-β activities.

The effects of IFN on individual cells are mediated through specific receptors on the cell membrane (types I and II), but the exact level of expression or function of these receptors in malignant cells is not known at present. In the present experiments, we

presented indirect evidence suggesting active participation of these receptors in GAC-2 cells. A synergistic antitumor effect was abolished by preincubation of IFN-β with anti-IFN-β mAb prior to cell culture (Fig. 3). This effect suggests that mAb neutralized the activity of IFN-β most probably by preventing it from binding to the receptor. A receptor for IFN-β is known to interact with IFN-α as a common receptor for type I IFNs. In the present experiment both IFNs showed identical synergistic effects on GAC-2 cells upon coculture with IFN-γ (Fig. 6A), but a short contact with IFN-β or -α caused a different degree of enhanced sensitivity to IFN-γ (Fig. 6B). Such discrepancy observed between coculture and pretreatment with type I IFNs might be ascribed to differences in the affinity of each IFN to type I IFN receptor [22]. Alternatively, the differences might be due to different levels of activity of other cofactors required for signal transduction [23, 24]. Thus, modulation of receptor activity could occur to control the level of signal transduction. To this effect, Schiller et al. [25] reported increased receptor binding and internalization of IFN-γ by IFN-β, but failed to explain the synergistic anti-proliferative effects of IFNs. On the other hand, Novelli et al. [26] described contrasting modulation of the IFN-γ receptor of human malignant T cells by environmental signals. Their results showed down-regulation of the receptor associated with enhancement of cell growth and up-regulation with apoptotic cell death.

Reduction of IFN-induced tyrosine phosphorylation correlates with the resistance of human melanoma cell lines to IFNs [27]. Since phosphorylated proteins correspond to components of the IFN signal transduction pathway and include, for example, receptors, tyrosine kinases and transcription factors, it is almost impossible at present to identify the protein(s) responsible for controlling the level of IFN sensitivity.

Recently Lee and Esteban [28] showed that transfection and expression of the IFN-induced double-stranded RNA-activated protein kinase (PKR), a serine/threonine kinase, in a human tumor cell line resulted in a rapid apoptotic cell death. Furthermore, Meurs and coworkers [29] suggested that PKR might function as a tumor suppressor gene based on the analysis of the relationship between its expression and tumor growth *in vivo*. The PKR is known to mediate antiviral activity and inhibit translation through phosphorylation of a transcription factor [30]. At present, it is considered an important candidate of key proteins that control cell death. Further investigation of the physiologic and pathophysiologic functions of proteins in the IFN signal transduction pathway and of individual IFN-regulated proteins is necessary in order to elucidate the mechanism(s) of antitumor activity of IFN.

Our results showed that cell death caused by IFNs was induced through apoptotic mechanisms. Characteristic nuclear morphology (Fig. 7B and C) was evident, and internucleosomal cleavage of DNA was subsequently demonstrated by electrophoresis (data not shown). Peculiar changes indicating leakage of nuclear fragments out of the cytoplasm were noted (Fig. 7C). Such a phenomenon may have occurred during the preparation of cytospin specimens and indicates profound membrane damage. We have so far not examined early changes in the membrane associated with apoptosis, but rather assessed cell death by trypan blue dye exclusion test. Although some cytotoxic drugs manifest their effect as necrosis in higher doses and apoptosis in lower doses, we postulate that the leaks in the plasma membrane occurred subsequently to apoptosis in IFN-treated GAC-2 cells. This is based on our observation that most dead GAC-2 cells observed in cultures with a high dose of one or more IFN showed fragmented nuclei

irrespective of membrane damage. At present, we are examining membrane pathophysiology in IFN-treated cells. Redistribution of the plasma membrane phospholipids, as reported by Martin et al. [31], would be quite important, since apoptosis or necrosis as a terminal stage is influenced by critical changes affecting the membrane structure.

The effect of IFN as a therapeutic agent against various neoplasms has been evaluated in several clinical trials. Actual application, however, is limited to certain types of cancers. Limitation stems not only from the primary effect but also from secondary adverse effects. Although we cannot extrapolate our *in vitro* results to *in vivo* conditions, several very important points became evident in our experiments. Augmentation of the cytocidal effect of IFN-γ is a more rational approach than intensifying the cytostatic activity of IFN-β in the use of IFN. The cytocidal effect of IFN-γ was irreversible even at a low dose in pretreated cells (Fig. 6, and our unpublished observations), whereas the effect of IFN-β in enhancing IFN-γ sensitivity was reversible (Fig. 5). This means that copresentation of both of IFNs to tumor tissues is required to induce the synergistic effect. Since a very short period of contact at a high dose or a longer incubation at a very low dose of IFN-β was effective in enhancing the sensitivity of tumor cells to IFN-γ, this goal could be achieved effectively by obtaining higher local concentrations of the drugs in conjunction with a tumor targeting system. This could eventually reduce the side effects by lowering the concentration of IFN in systemic circulation.

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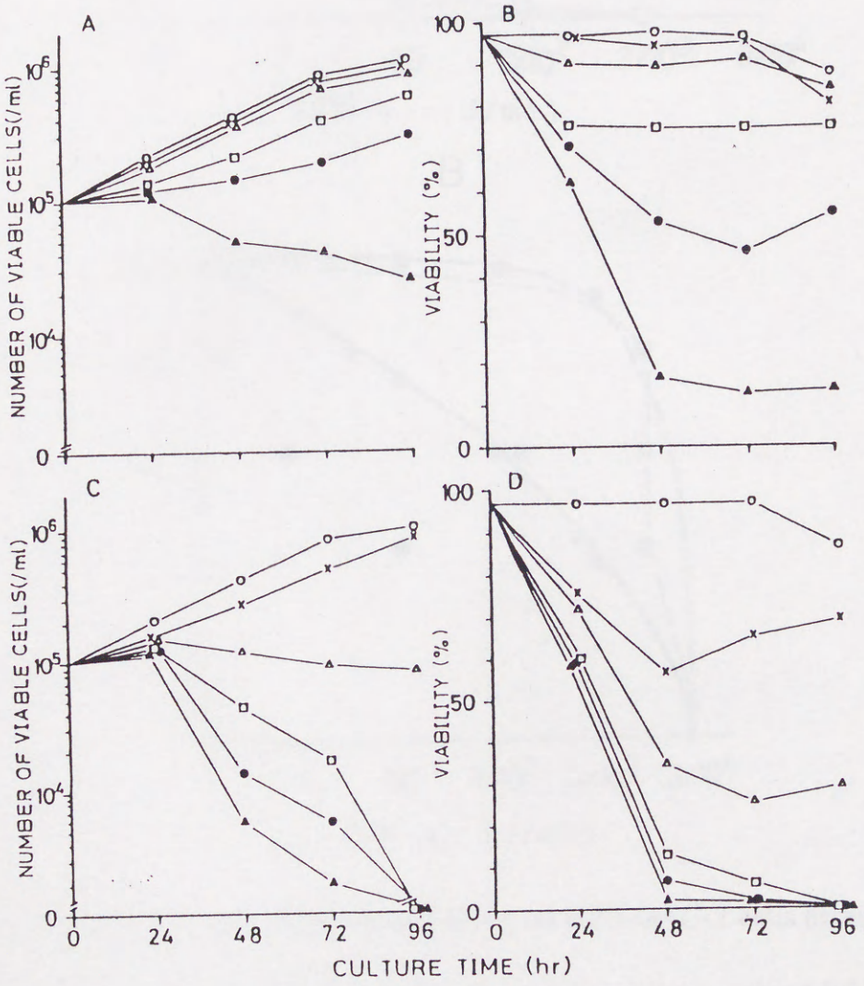


Figure 1. Growth kinetics (A, C) and cell viability (B, D) of HPE-GAC-2 cells cocultured with IFN-β (A, B) or IFN-γ (C, D). *Open circles*, no IFN; *x*, 3; *open triangles*, 30; *open squares*, $3x10^2$; *closed circles*, $3x10^3$; *closed triangles*, $3x10^4$ IU/ml or U/ml of IFN-β or -γ, respectively. Each point represents the mean of two cultures, and is representative data of three independent experiments as described in the Materials and Methods.

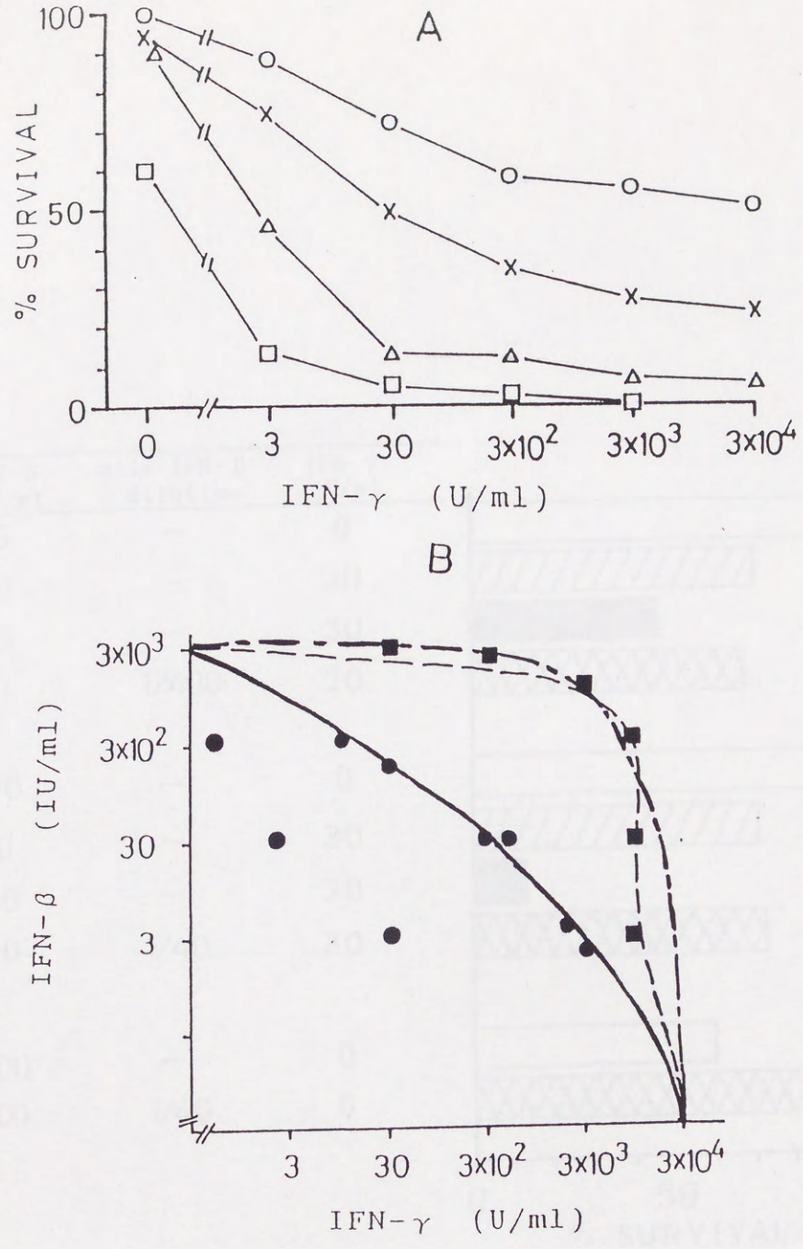


Figure 2. Combined effects of IFN-β and IFN-γ on HPE-GAC-2 cells assessed at 24 hr of culture. A, percent survival compared with a control culture without IFN. Abscissa, log concentration of IFN-γ, *open circles*, no IFN-β; *x*, 3; *open triangles*, 30; *open squares*, $3x10^2$ IU/ml of IFN-β. B, analysis of synergy of data shown in (A) by isobologram at IC_{50}. Abscissa, log concentration of IFN-γ, with IC_{50} at $3x10^4$ IU/ml. Ordinate, log concentration of IFN-β, with IC_{50} at $3x10^3$ U/ml, ——●——, mode I line; —— – —■— – , mode II (IFN-γ) line; – – – – ■ – , mode II (IFN-β) line; ● , experimental data relating to the combination of IFN-β and IFN-γ.

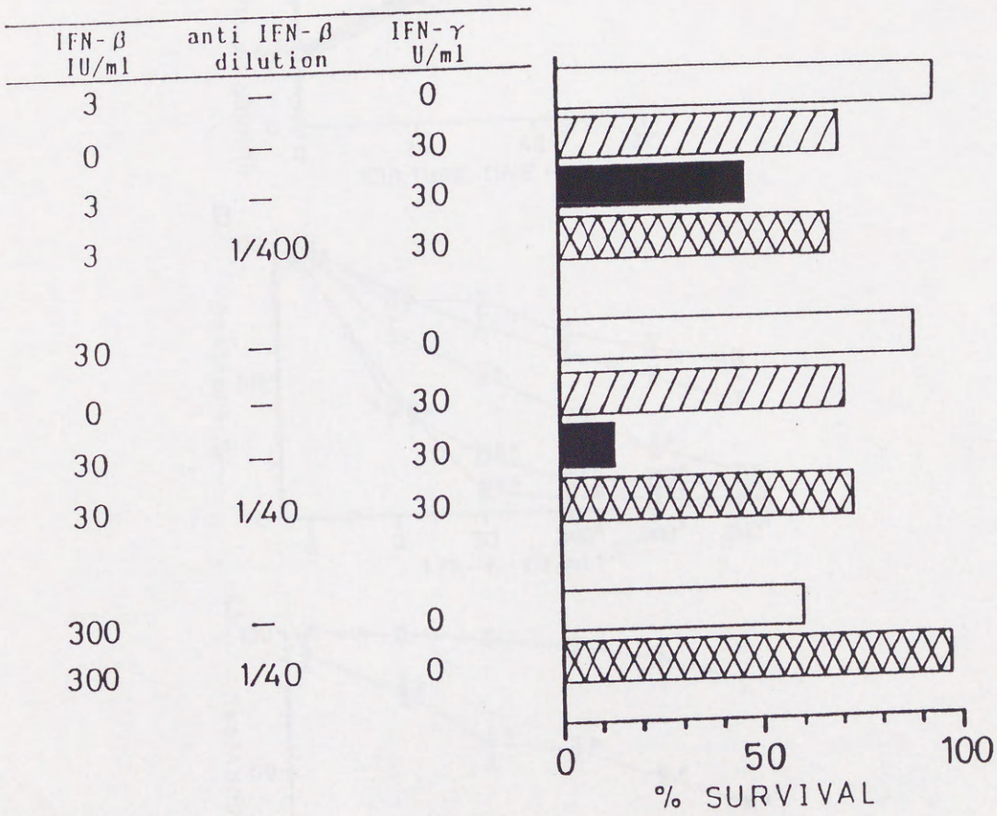


Figure 3. Effect of preincubation of IFN-β with anti-IFN-β monoclonal antibody on the subsequent coculture with HPE-GAC-2 cells assessed at 24 hr of coculture. Open bars, culture with IFN-β alone; shaded bars, culture with IFN-γ alone; solid bars, culture in combination of IFN-β and IFN-γ; crosshatched bars, IFN-β pretreated with the antibody before commencement of culture.

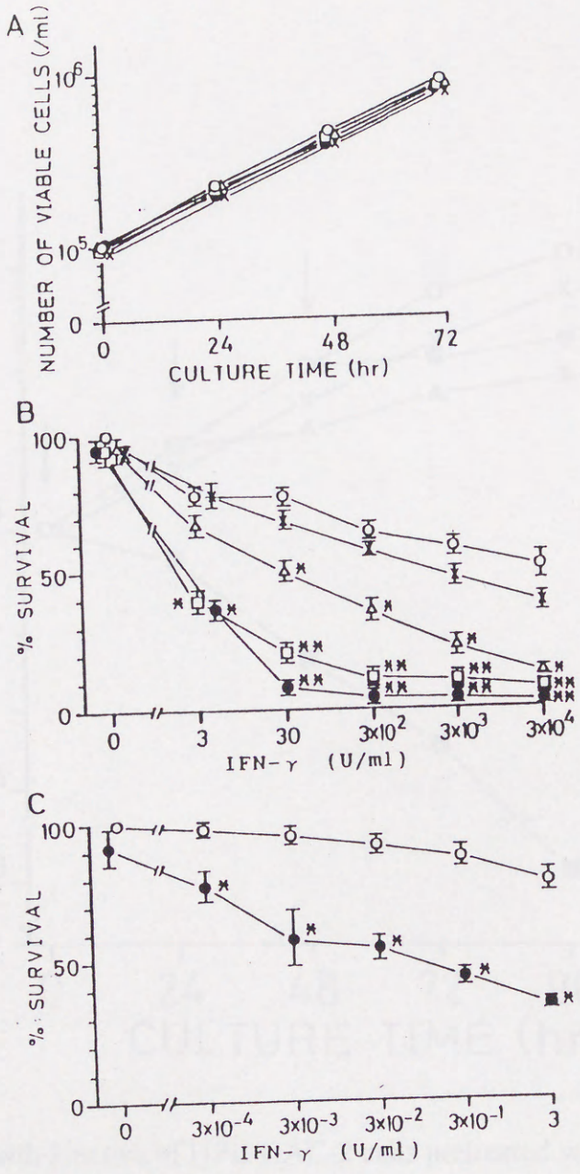


Figure 4. Effect of pretreatment of HPE-GAC-2 cells with IFN-β on subsequent culture without IFN (A) or with IFN-γ assessed at 24 hr of culture (B, C). Abscissa; A, culture period; B and C, log concentration of IFN-γ. Pretreatment of cells; *open circles*, medium only; *x*, 30; *open triangles*, 3×10^2; *open squares*, 3×10^3; *closed circles*, 3×10^4 IU/ml of IFN-β. *, **; Differences are significant, $p<0.01$ or $p<0.001$, respectively.

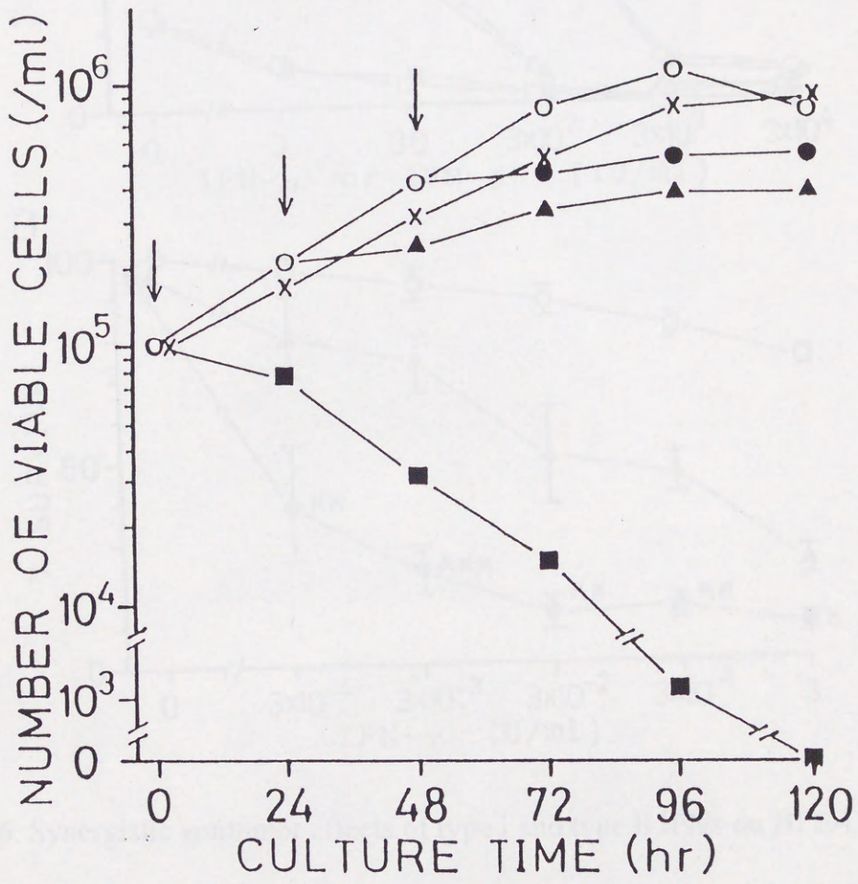


Figure 5. Growth kinetics of HPE-GAC-2 cells pretreated with IFN-β, and cocultured with IFN-γ added at different intervals after commencement of culture. The cells were pretreated with media alone (*x*) or with $3x10^4$ IU/ml of IFN-β (*open circles*). Three U/ml of IFN-γ was added to both control and IFN-β treated cells at 0 (*closed squares, x*), and to IFN-β treared cells at 24 (*closed triangles*) or 48 hr (*closed circles*) as indicated by arrows.

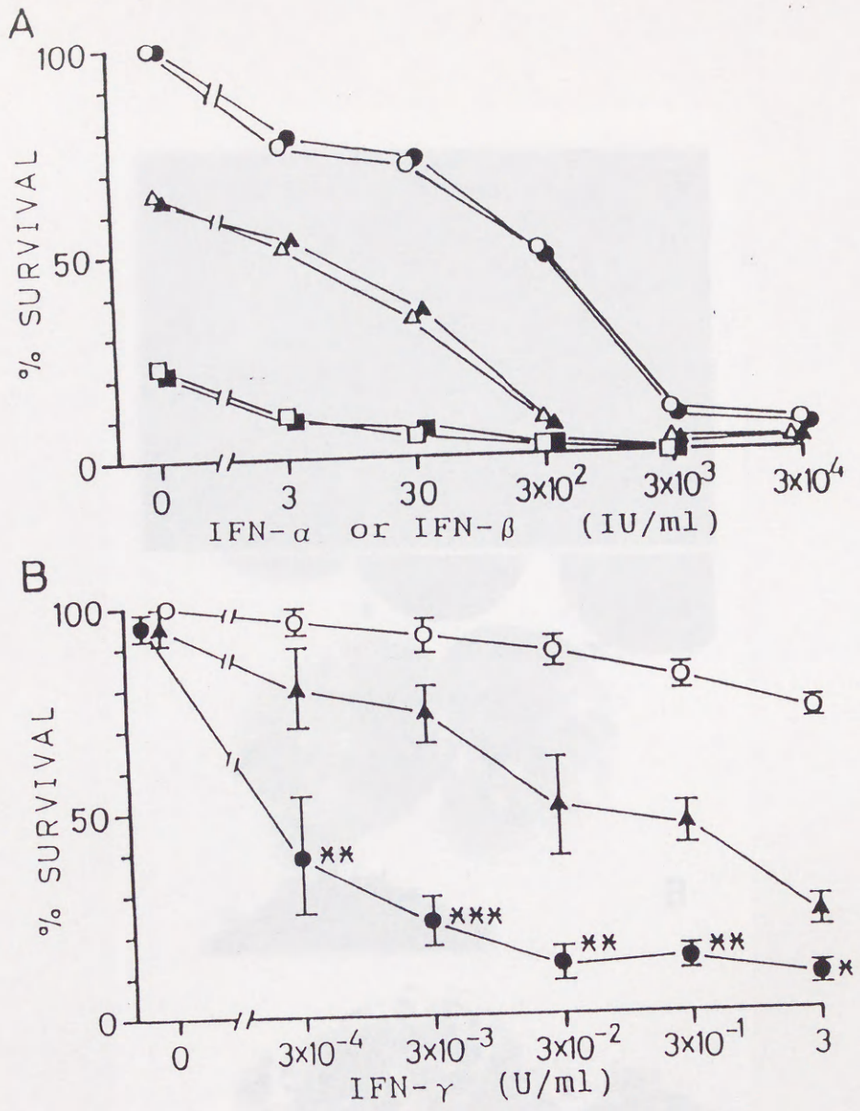


Figure 6. Synergistic antitumor effects of type I and type II IFNs on HPE-GAC-2 cells. A. The cells were cocultured with varying concentrations (abscissa) of IFN-α (*open symbols*) or -β (*closed symbols*) with 0 (*circles*), 3 (*triangles*) or 30 (*squares*) U/ml of IFN-γ for 72 hr. B. The cells were pretreated with medium alone (*open circles*), or $3x10^4$ IU/ml of IFN-α (*closed triangles*) or IFN-β (*closed circles*), washed and subsequently cocultured with varying concentrations of IFN-γ (abscissa) for 48 hr. The Difference in the percentage of survival between cells pretreated with IFN-α and IFN-β , and then cultured with IFN-γ , was significant by student's t-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

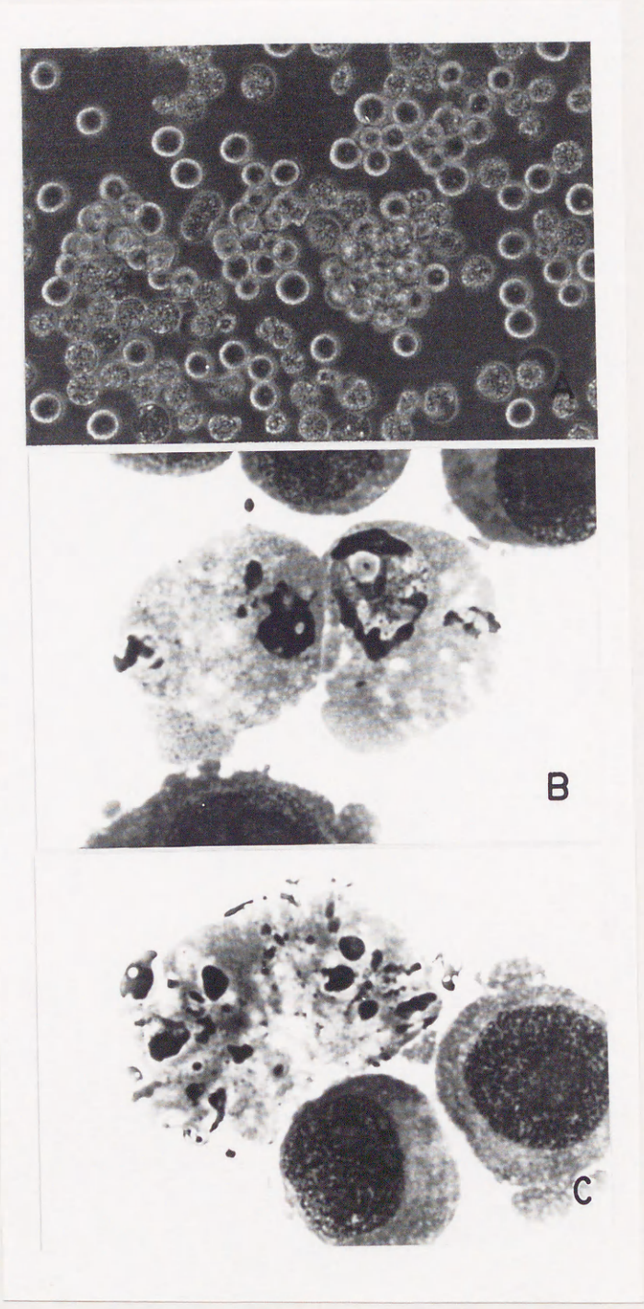
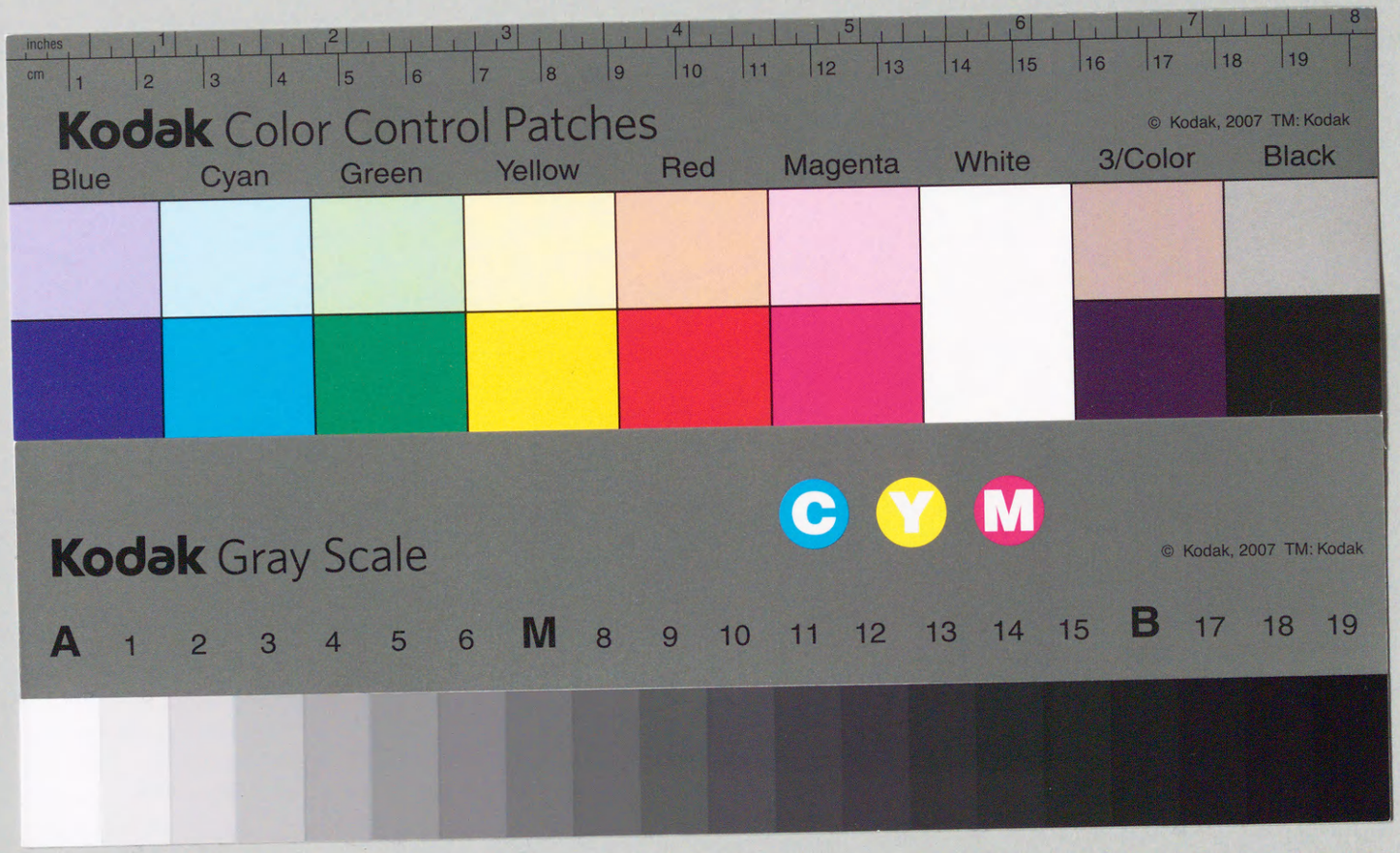


Figure 7. Morphology of IFN-treated cells by phase contrast microscopy (A x50) and May Gruenwald and Giemsa staining (B and C, x500).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Cyan
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Red
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White
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