

23. Cross F, Roberts J, Weintraub H. Simple and complex cell cycles. *Annu Rev Cell Biol* 1989, 5, 341–395.
24. Wiernik PH, Schwartz EL, Strumann JJ, Dutcher JP, Lipton RB, Paietta E. Phase I clinical and pharmacokinetic study of taxol. *Cancer Res* 1987, 47, 2486–2493.
25. Markman M, Rowinsky E, Hakes T, *et al.* Phase I trial of intraperitoneal taxol: a Gynecologic Oncology Group study. *J Clin Oncol* 1992, 10, 1485–1491.
26. Kestell P, Paxton JW, Evans PC, *et al.* Disposition of amsacrine and its analogue 9-((2-methoxy-4-[(methylsulfonyl)-amino]phenyl)amino)-N,5-dimethyl-4-acridine-carboxamide (CI-921) in plasma, liver, and Lewis lung tumors in mice. *Cancer Res* 1990, 50, 503–508.
27. Rowinsky EK, Gilbert MR, McGuire WP, *et al.* Sequences of taxol and cisplatin: a phase I and pharmacologic study. *J Clin Oncol* 1991, 9, 1692–1703.
28. Jekunen AP, Christen RD, Shalinsky DR, Howell SB. Synergistic interaction between cisplatin and taxol in human ovarian carcinoma cells *in vitro*. *Br J Cancer* 1994, 69, 299–306.

**Acknowledgements**—This work was supported by the Cancer Society of New Zealand, by its Auckland Division, and by the Health Research Council of New Zealand. The authors would like to thank the patients who allowed samples of their tumours to be used for this research project.



Pergamon

*European Journal of Cancer* Vol. 31A, No. 2, pp. 237–243, 1995  
Copyright © 1995 Elsevier Science Ltd  
Printed in Great Britain. All rights reserved  
0959-8049/95 \$9.50+0.00

0959-8049(94)00481-1

## Growth Regulation by All-trans-retinoic Acid and Retinoic Acid Receptor Messenger Ribonucleic Acids Expression in Gastric Cancer Cells

R.-Y. Shyu, S.-Y. Jiang, S.-L. Huang, T.-C. Chang, K.-L. Wu, S.R. Roffler and M.-Y. Yeh

Retinoic acid has been recognised as a pivotal compound in cell differentiation, proliferation and malignant transformation. We investigated the effects of all-trans-retinoic acid on cell growth and the expression of retinoid nuclear receptor mRNAs in gastric cancer cells *in vitro*. Cell growth was quantified by measuring total cellular DNA. The growth of two of the five gastric cancer cell lines tested (SC-M1 and TSGH9201) was inhibited by all-trans-retinoic acid at concentrations ranging from  $1 \times 10^{-8}$  M to  $1 \times 10^{-6}$  M. Growth inhibition was associated with G0/G1 phase arrest as determined by flow cytometric analysis. Northern blot analysis showed that all five cell lines expressed mRNA for retinoic acid receptors  $\alpha$  and retinoic x receptor  $\alpha$  and  $\beta$ . Retinoic acid receptor  $\beta$  mRNA was only expressed in TSGH9201 and TMK-1 gastric cancer cell lines. Two RAR $\gamma$  mRNA transcripts (3.2 and 3.0 kb) were detected in SC-M1 and TSGH9201 cells. RA-resistant cells had markedly decreased levels of the 3.2 kb RAR $\gamma$  transcript. All-trans-retinoic acid had a cytostatic effect on the growth of some gastric cancer cells, which may be associated with the expression of retinoic acid receptors.

**Key words:** retinoic acid, retinoic acid receptor, gastric cancer, growth regulation

*Eur J Cancer*, Vol. 31A, No. 2, pp. 237–243, 1995

### INTRODUCTION

THE RETINOIDS are a pharmacological class consisting of vitamin A (retinol) and related derivatives, such as retinoic acid (RA), that display a wide range of biological functions in cellular differentiation, proliferation, vision, immune function and malignant transformation [1]. Recent reports of dramatic antitumour effects by RA on acute promyelocytic leukaemia as well as squamous cell cancer of the uterine cervix and the skin have encouraged the exploration of the use of retinoids in the therapy of various types of cancer.

The effects of RA are mediated through specific receptors. Two structurally related retinoid nuclear receptors exist: retinoic acid receptors (RARs) and retinoic x receptors (RXRs). Three

types ( $\alpha$ ,  $\beta$  and  $\gamma$ ) of receptors have been identified for each of them [2–7]. All-trans-retinoic acid (tRA) is a ligand for RARs. However, 9-cis RA is a bifunctional ligand that binds both RAR and RXR [8]. All the receptors belong to the steroid/thyroid superfamily. Their expression is tissue specific and developmentally regulated. RARs and RXRs consist of six distinct functional domains, designated A to F [9]. The DNA binding C domain and the RA binding E domain are highly conserved between receptors. RARs are thought to activate gene expression by binding to direct repeats of pentameric sequences called retinoic acid response elements. Genes such as RAR $\beta$  and alcohol dehydrogenase are known to be regulated by RA [1]. Control of growth and differentiation pathways may be mediated in part by

interactions at the retinoic acid response element level among RARs, RXRs or other transcription factors, which often induce cellular differentiation and inhibit cell growth [1].

Studies *in vitro* have shown that retinoids display a wide spectrum of antitumour activities on acute promyelocytic leukaemia, teratocarcinoma, neuroblastoma, melanoma and breast cancer, etc. [1, 10–12]. The mechanism of antitumour activity may be directly related to induction of cell differentiation or growth inhibition. Growth inhibition was associated with G0/G1 arrest in several studies [13]. However, the induction of programmed cell death (apoptosis) has also been observed [14].

Gastric cancer is a common disease throughout the world. The prognosis for most patients with gastric cancer remains poor due to lack of effective therapeutic agents. Therefore, it is important to search for new treatment modalities. The clinical significance of retinoids in the treatment and prevention of gastric cancer has been studied. Haenszel and associates have demonstrated that decreased levels of  $\beta$ -carotene are associated with increased risk of gastric cancer [15]. In addition, gastric cancer patients have lower serum vitamin A levels than the normal population [16]. Carotenoid has been shown to prevent the development of chemically induced gastric cancer [17]. Finally, combination treatment with vitamin A, OK432, mitomycin C and tagafur resulted in decreased recurrence of gastric cancer following primary surgery compared to patients that did not receive vitamin A treatment [18]. Therefore, the potential of vitamin A or RA in the prevention of adjuvant therapy of gastric cancer is promising. However, there are no studies in which the mechanisms by which RA regulates the growth of gastric cancer cells have been investigated.

We studied the effects of the active metabolite of RA, tRA, on the growth of five gastric cancer cells *in vitro*. We observed that tRA inhibited the growth of two of the five cancer cell lines. Growth inhibition was associated with arrest of the cell cycle in the G0/G1 phase. The expression of RAR and RXR mRNAs and their association with RA sensitivity were further analysed.

## MATERIALS AND METHODS

### Cell culture

Human gastric cancer cell lines, SC-M1, TMC-1, TSGH9201, TMK-1 and AGS, were maintained in RPMI 1640 medium supplemented with 25 mM HEPES, 26 mM NaHCO<sub>3</sub>, 2 mM glutamine, 100 units per ml penicillin, 100  $\mu$ g per ml streptomycin and 10% fetal bovine serum (FBS) at 37°C with 5% CO<sub>2</sub>. SC-M1 cells were obtained from Dr C.-L. Meng (National Defense Medical Center, Taipei, Taiwan, Republic of China) [19]. TMC-1 cells, derived from the lymph node metastasis of a moderately differentiated gastric adenocarcinoma, were obtained from Dr T.-M. Chang (Tri-Service General Hospital, Taipei, Taiwan). TSGH9201 cells, derived from the ascites of a patient presenting signet ring cell carcinoma of the stomach, were established in our laboratory. TMK-1 cells were obtained

from Dr E. Tahara (Hiroshima University, Hiroshima, Japan) [20]. AGS cells were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). All tissue culture reagents and medium were obtained from Gibco (Gaithersbury, Maryland, U.S.A.). Cells were free of mycoplasma using Hoechst 33258 dye (Sigma Chemical Co., St Louis, Missouri, U.S.A.). To investigate the effects of tRA on cell growth, media containing 5% serum treated with dextran-coated charcoal (to remove steroid hormones) or without charcoal treatment were used for cell plating and during tRA addition. Cells were cultured in serum without charcoal treatment for all other experiments. tRA was dissolved in absolute ethanol under subdued light. The final concentration of ethanol in the media was 0.1%. All tissue culture experiments were conducted under yellow light to decrease tRA inactivation. The morphology of cells was recorded with a Nikon TMD phase contrast inverted microscope (Tokyo, Japan). The viability of cells was determined by the trypan blue dye exclusion method.

### Growth response assay

Cells were plated in triplicate in 25 cm<sup>2</sup> flasks or 24-well plates for 2 days. Media containing tRA or ethanol (0.1%) only was then added for the indicated period with media changes performed every other day. Cells were harvested and sonicated in calcium and magnesium free Hanks' balanced salt solution. Total cellular DNA, measured using Hoechst 33258 dye, was used as a measure of cell growth [21]. The intensity of fluorescence was recorded using a model 450 fluorometer (Sequoia-Turner, Mountain View, California, U.S.A.). Two to five independent experiments for each cell line were conducted, and representative results are presented. Each data point represents the mean of triplicate samples, and error bars show standard errors of the mean.

### Cell cycle analysis

Cells plated in 15 cm dishes were cultured in media containing 5% FBS and tRA or ethanol for 6 days. Cells in logarithmic growth were labelled with 5  $\mu$ M bromodeoxyuridine (BrdUrd) for 20 min. Cells were then harvested and fixed in 70% ethanol. Nuclei were prepared by incubating cells in 0.04% pepsin, 0.1 N HCl for 20 min at room temperature followed by addition of 2 N HCl at 37°C for 30 min [22]. Following neutralisation with 0.1 M sodium borate, nuclei were stained with anti-BrdUrd monoclonal antibody (Becton-Dickinson) at room temperature for 45 min and fluorescein labelled goat anti-mouse antibody (1:50) (Sigma) for 30 min. Nuclei were then stained with 10  $\mu$ g/ml propidium iodide, 5  $\mu$ g/ml RNase A overnight at 4°C and analysed by FACScan (Becton-Dickinson). The nuclei were excited at 488 nm with simultaneous list mode acquisition of both the green fluorescence from fluorescein (515–560 nm) and the red fluorescence from propidium iodide (>620 nm) as a measurement of incorporated BrdUrd and total DNA content, respectively. The fraction of cells in G0/G1, S and G2/M phases were analysed using the LYSYS program.

### RNA preparation and Northern blot analysis

Poly (A)<sup>+</sup> RNA was prepared as described by Badley and colleagues [23]. Cells were lysed in buffer containing 0.2 M NaCl, 0.2 M Tris-HCl pH 7.5, 1.5 mM MgCl<sub>2</sub>, 2% SDS, 200  $\mu$ g/ml protease K and 50  $\mu$ M aurintricarboxylic acid (Sigma) and incubated at 45°C for 2 h. Cell lysates were then incubated with oligo dT cellulose (Boehringer Mannheim, Germany) in the same buffer containing 0.5 M NaCl at room

Correspondence to M.-Y. Yeh at the National Defense Medical Center, Department of Microbiology and Immunology, P.O. Box 90048–505, Taipei, Taiwan, Republic of China. R.-Y. Shyu, S.-Y. Jiang and T.-C. Chang are at the Graduate Institute of Medical Sciences, National Defense Medical Center; S.-Y. Jiang, S.-L. Huang and M.-Y. Yeh are at the Cancer Research Laboratory, R.-Y. Shyu and K.-L. Wu are at the Department of Internal Medicine, Tri-Service General Hospital; S.R. Roffler is at the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China.

Supported in parts by grants from National Science Council, Department of Health and Academia Sinica, Republic of China.

Revised 8 Oct. 1994; accepted 13 Oct. 1994.

temperature for 1 h on a rotatory shaker. After washing, RNA was eluted with 0.01 M Tris-HCl, pH 7.5. RNA was then fractionated on a 1.1% agarose, 1.1% formaldehyde gel in 5 mM NaOAc, 1 mM EDTA, 20 mM 3-[N-Morpholino]propanesulphonic acid (Sigma), pH 7.0 and transferred to a nylon membrane by capillary blotting in  $20 \times$  SSC (3 M NaCl, 0.3 M Na<sub>3</sub> citrate, pH 7.0). Blots were UV-fixed, prehybridised and hybridised at 42°C in buffer containing 50% (v/v) formamide,  $5 \times$  SSC, 2% (w/v) blocking reagent, 0.1% N-lauroylsarcosine and 0.2% (w/v) SDS. The membranes were washed with  $2 \times$  SSC containing 0.1% SDS and then washed with  $0.1 \times$  SSC containing 0.1% SDS at 68°C for 30 min. Specific hybridisation was detected by a DIG luminescent detection kit using lumigen-PPD as the substrate and was recorded using Kodak XAR-5 film at room temperature. The intensity of luminescence was maximal 7–17 h after substrate activation. The RXR $\alpha$  membrane was exposed to X-ray film for 20 min whereas  $\beta$ -actin membranes were exposed for 2–30 min immediately after substrate activation. RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$ , RXR $\beta$  and RXR $\gamma$  membranes were exposed for 11, 11, 30, 90, and 120 min, respectively, after the substrate has been activated for 15 h. To prepare membranes for rehybridisation, membranes were incubated with 0.05 M Tris-HCl, pH 8.0, 1% SDS and 50% formamide at 68°C for 1 h and then prehybridised as described above.

The Hind III digested 1.8 kb cDNA probe encoding *Drosophila* actin derived from 1DmA2 was isolated from plasmid actin #6 [24]. The EcoRI digested 1.6 kb cDNA encoding the mouse RAR $\alpha$  was isolated from pSG5-mRAR $\alpha$  [2]. The EcoRI digested 0.6 kb cDNA encoding the human RAR $\beta$  was isolated from B1-RAR [3]. The Bam HI digested 1.8 kb cDNA encoding the human RAR $\gamma$  was isolated from hRAR $\gamma$ . $\phi$  [4]. The Eco RI digested 1.9 kb cDNA encoding the human RXR $\alpha$  was isolated from A5C/hRXR $\alpha$  [5]. The Eco RI digested 2.2 kb cDNA encoding the human RXR $\beta$  was isolated from mRXR $\beta$  (H-2RIIBP) [6]. The Eco RI digested 1.6 kb cDNA encoding the mouse RXR $\gamma$  was isolated from pBSK-mRXR $\gamma$  [7]. cDNAs were labelled with digoxigenin using a DNA labelling kit. The levels of specific RNA were quantitated using a 300S computing densitometer (Molecular Dynamics, Sunnyvale, California, U.S.A.) and analysed by Image Quant Software. The relative levels of RAR $\alpha$  (3.5 and 2.9 kb), RAR $\beta$  (3.4 and 3.1 kb), RAR $\gamma$  (3.2 and 3.0 kb), RXR $\alpha$  (5.6 kb) and RXR $\beta$  (3.0 kb) were normalised to the levels of  $\beta$ -actin mRNA of each individual cell line from the same nylon membrane.

## RESULTS

### *Growth inhibition and morphological alteration of gastric cancer cells by tRA*

The effects of tRA on the growth of SC-M1, TSGH9201, AGS, TMK-1 and TMC-1 cells were determined by measuring total cellular DNA. Exposure to tRA for 6 days in media containing 5% FBS inhibited the growth of SC-M1 and TSGH9201 cells in a concentration dependent manner (Figure 1). Growth of SC-M1 or TSGH9201 cells was inhibited by a maximum of 77 or 50% of control levels, respectively, at a concentration of  $5 \times 10^{-6}$  M tRA. SC-M1 cells were the most sensitive of the cell lines tested to growth inhibition by tRA. The growth of AGS cells was significantly inhibited by tRA only at a concentration of  $5 \times 10^{-6}$  M. tRA had no effect on the growth of TMK-1 and TMC-1 cells at all tested concentrations. Similar results were observed for all five gastric cancer cell lines when they were cultured in medium containing FBS that had been

treated with dextran-coated charcoal to remove retinoids and other steroid hormones (data not shown).

The effects of tRA on SC-M1 cell growth were further analysed by exposing cells for different periods to various concentrations of tRA (Figure 2). As described above, tRA inhibited the growth of SC-M1 cells in a concentration dependent manner;  $1 \times 10^{-9}$  M of tRA had no effect on cell growth whereas a growth inhibitory effect at high tRA concentrations ( $1 \times 10^{-6}$  M and  $5 \times 10^{-6}$  M) was evident after 3–4 days of treatment. Increased growth inhibition was observed with increasing concentrations and length of tRA incubation.

No apparent morphological alteration was observed in TSGH9201 cells following tRA treatment for 6 days. However, SC-M1 cells became flattened and enlarged. Increased numbers of processes over the cell surface were observed after 16 days of treatment (Figure 3). The physical properties of SC-M1 cells were also altered by tRA. Cells became firmly attached to the solid phase following tRA treatment.

The growth inhibition and morphological alterations induced by tRA in SC-M1 cells were largely reversible. After culturing SC-M1 cells in tRA ( $5 \times 10^{-6}$  M) for 70 days, cells that were transferred to tRA-free medium had an increased growth rate compared to the cells that were continuously cultured in media containing tRA (Figure 4). Increased cell numbers were observed 5 days after tRA removal. Cell viability was greater than 95% regardless of whether tRA was removed or not. Although tRA removal increased the growth rate of SC-M1 cells, it is of note that the rate of cell growth was slower than SC-M1 cells that had not been exposed to tRA. After tRA removal, the morphology of SC-M1 cells gradually reversed to their original appearances of small cell size, fewer processes and evidence of a cell border. A minority of cells, however, continued to exhibit altered morphology after tRA removal.

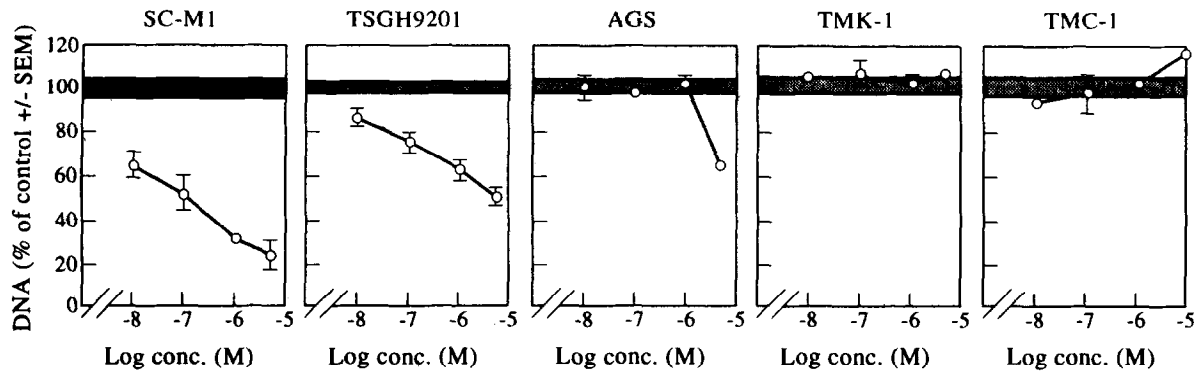
### *Effects of tRA on the cell cycle phase distribution*

The effects of tRA on the cell cycle phase distribution were analysed by flow cytometry. Total DNA was detected by staining with propidium iodide whereas cells in the S phase were detected by BrdUrd incorporation and staining with an anti-BrdUrd antibody. In unsynchronised SC-M1 cells, tRA at concentrations of  $1 \times 10^{-8}$  M and  $1 \times 10^{-6}$  M increased the fraction of cells in G0/G1 phase and decreased the fraction of cells in the S phase in a concentration related manner (Figure 5). Similar results were not observed in the G2/M phase. Increased G0/G1 and decreased S phase fractions were also observed, to a lesser extent, in the other tRA-sensitive cell line TSGH9201 (data not shown). tRA had no effect on the cell cycle phase distribution of RA-resistant TMK-1 cells (Figure 5).

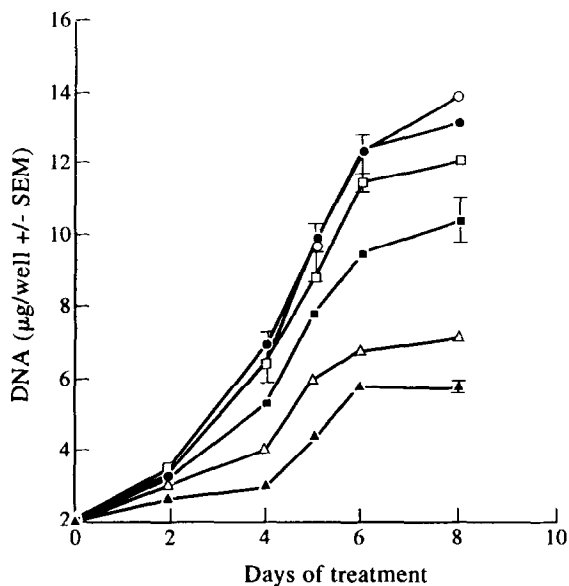
### *Northern blot analysis of the basal levels of RARs and RXRs mRNAs*

The basal levels of RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  mRNAs in cells cultured in regular media were analysed using Northern blot analysis. All five gastric cancer cells expressed RAR $\alpha$  mRNA with molecular sizes of 3.5 and 2.9 kb (Figure 6). The 4.8 kb band may be derived from cross hybridisation with 28S ribosomal RNA. Only TSGH9201 and TMK-1 cells expressed RAR $\beta$  mRNA with molecular sizes of 3.4 and 3.1 kb. Densitometric analysis showed that TSGH9201 cells had approximately five times more RAR $\beta$  mRNA than TMK-1 cells (Table 1). Two RAR $\gamma$  transcripts (3.2 and 3.0 kb) were detected. SC-M1 and TSGH9201 cells expressed both transcripts as a broad band. However, AGS cells only expressed the 3.2 kb transcript

(a)



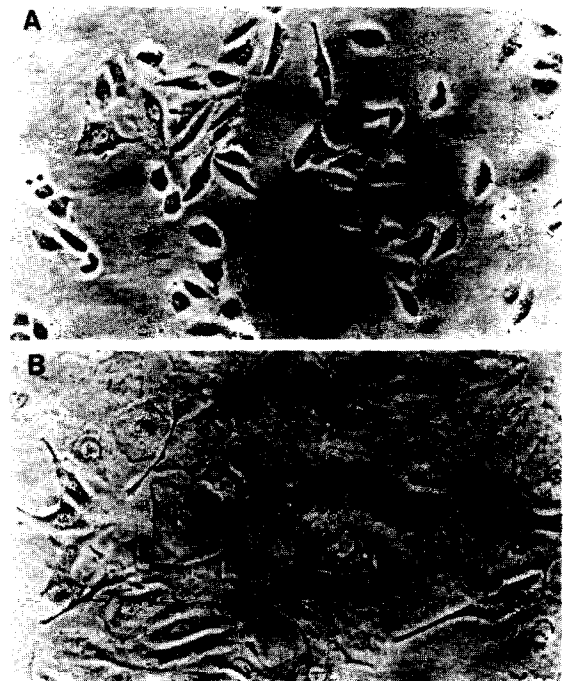
**Figure 1.** Effect of various concentrations of tRA on the growth of gastric cancer cells. Cells were plated in triplicate for 2 days and then cultured in media containing 5% FBS and various concentrations of tRA or control media for 6 days. The levels of cell growth in control media are represented as shaded bars. Standard errors of the mean are shown.



**Figure 2.** Effect of tRA exposure time on the growth of SC-M1 cells. Cells were plated in 24-well plates for 2 days and then cultured in media containing 5% FBS and various concentrations of tRA for the indicated times. ○, control; ●, tRA  $1 \times 10^{-9}$  M; □, tRA  $1 \times 10^{-8}$  M; ■, tRA  $1 \times 10^{-7}$  M; △, tRA  $1 \times 10^{-6}$  M; ▲, tRA  $5 \times 10^{-6}$  M.

whereas TMK-1 cells only expressed the 3.0 kb transcript. Neither the 3.2 nor 3.0 kb RAR $\gamma$  transcript was observed in TMC-1 cells. Further analysis using the polymerase chain reaction and primers corresponding to the A and B domains of RAR $\gamma$  [25] detected the expression of RAR $\gamma$  mRNA in TMC-1 cells (data not shown). When the levels of RAR $\alpha$  (3.5 and 2.9 kb), RAR $\beta$  (3.4 and 3.2 kb) and RAR $\gamma$  (3.2 and 3.0 kb) mRNA were normalised to the  $\beta$ -actin levels after densitometric analysis, SC-M1 cells, which were most sensitive to tRA, were found to express the highest levels of both RAR $\alpha$  and RAR $\gamma$  mRNA (Table 1). TSGH9201 cells, which were also sensitive to the growth inhibitory effects of tRA, also expressed higher levels of RAR $\alpha$  and RAR $\gamma$  than did tRA insensitive cell lines.

All five cell lines expressed both RXR $\alpha$  and RXR $\beta$  mRNA (Figure 7). RXR $\gamma$  mRNA, however, was not detectable in any of the tested gastric cancer cell lines. RXR $\alpha$  mRNA was much more abundant than RXR $\beta$  mRNA. Densitometric analysis of

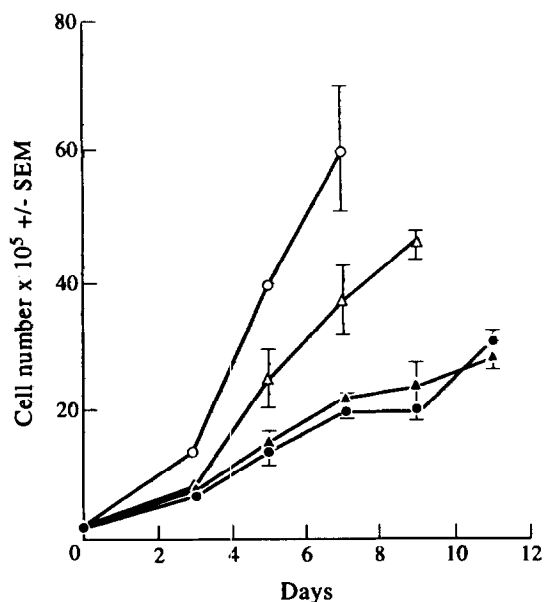


**Figure 3.** Effect of tRA on the morphology of SC-M1 cells. Cells were cultured in medium containing 5% FBS and 0.1% ethanol (A) or  $5 \times 10^{-6}$  M tRA (B) for 16 days. Phase contrast  $\times 200$ .

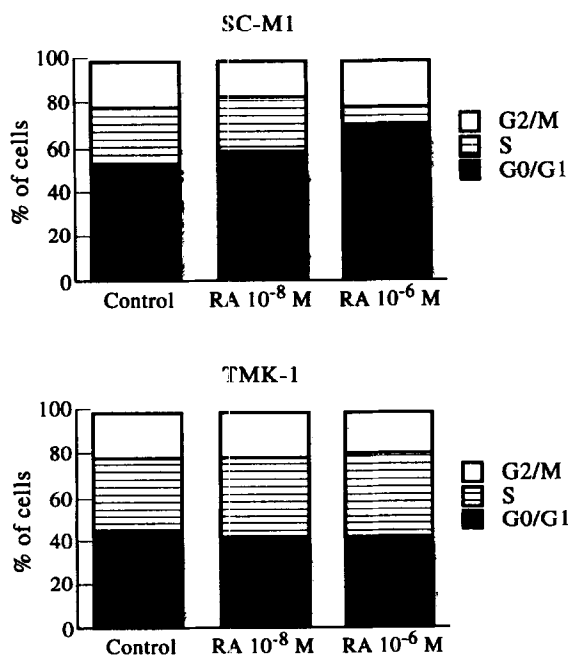
the Northern blot data showed that tRA sensitive TSGH9201 and SC-M1 cells expressed the highest levels of RXR $\alpha$  mRNA (Table 1). No correlation was apparent between tRA sensitivity and RXR $\beta$  expression.

## DISCUSSION

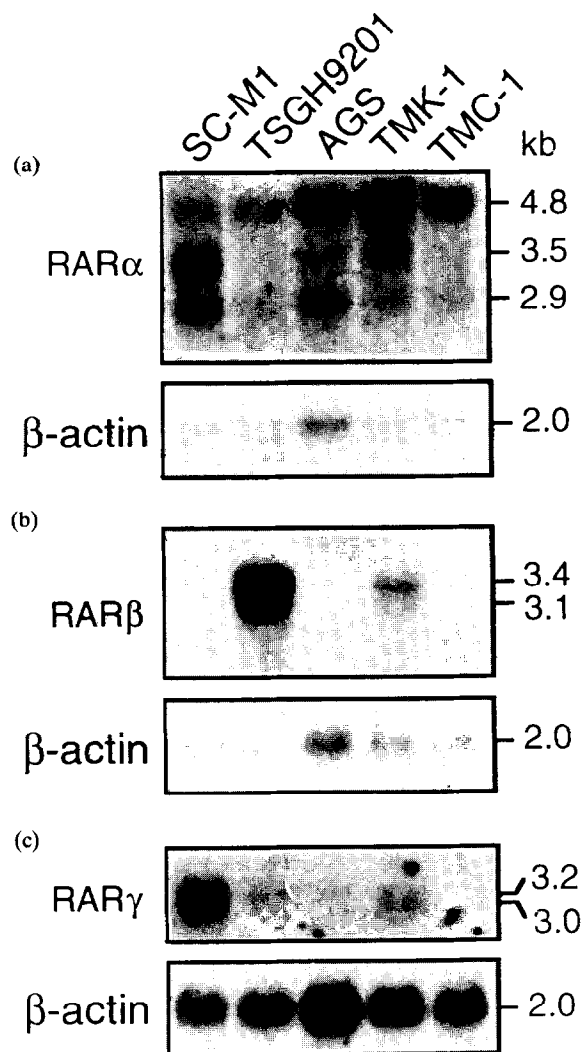
Retinoids have been found to inhibit the growth of several types of tumour cells *in vitro* [1, 10–12]. We investigated the effects of tRA on the growth of five gastric cancer cell lines. The results show that the growth of SC-M1 and TSGH9201 cells were sensitive to tRA. AGS cells were partially sensitive whereas TMK-1 and TMC-1 cells were resistant. Growth inhibition was associated with G0/G1 arrest in RA-sensitive cell lines. The expression of RARs and RXRs were further investigated. All five cell lines expressed different levels of RAR and RXR mRNA. The most sensitive cell line SC-M1 expressed the



**Figure 4.** Reversal of tRA inhibition of SC-M1 cell growth. Cells without (○ and ●) and with (△ and ▲) previous tRA ( $5 \times 10^{-6}$  M) treatment for 70 days were plated in 6 well plates at densities of 1 or  $3 \times 10^5$  cells per flask, respectively, for 2 days in RPMI 1640 medium containing 5% FBS. Cells were treated with medium containing 0.1% ethanol (open symbols) or medium containing  $5 \times 10^{-6}$  M tRA (closed symbols) for the indicated periods with media changes every 2 days. Cells were trypsinised and counted using the trypan blue dye exclusion method.



**Figure 5.** Effect of tRA on the cell cycle phase distribution of unsynchronised SC-M1 and TMK-1 cells.

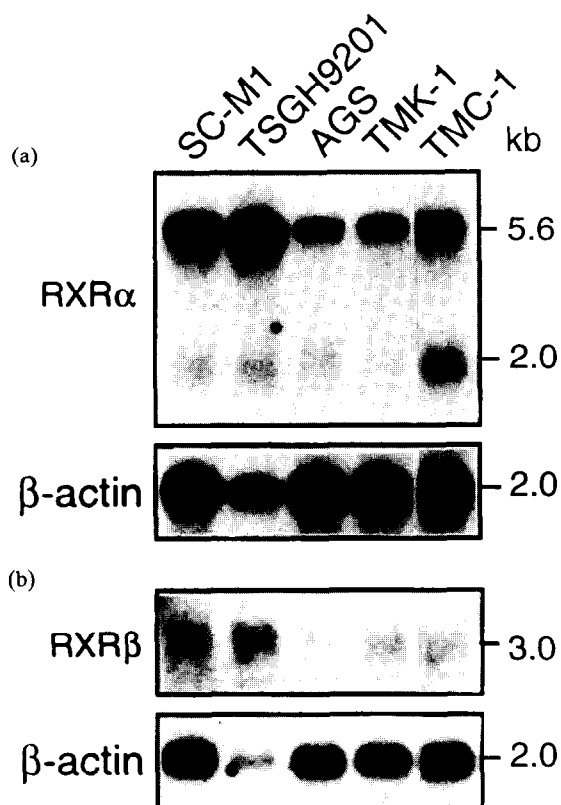


**Figure 6.** Expression of RAR mRNAs in stomach cancer cells. Poly (A)<sup>+</sup> RNA ( $10 \mu\text{g}$  per lane) was hybridised with digoxigenin-labelled cDNA for RAR $\alpha$  (a), RAR $\beta$  (b) or RAR $\gamma$  (c). Probes were removed and membranes were rehybridised with a  $\beta$ -actin probe as shown on the lower section of each panel.

**Table 1.** Relative levels of RAR and RXR mRNA expression in gastric cancer cell lines. X-ray films shown in Figures 6 and 7 were scanned with a densitometer. The levels of each subtype of RAR and RXR mRNAs were normalised to the  $\beta$ -actin levels as described in Materials and Methods. The relative levels of RAR $\alpha$ , RAR $\gamma$ , RXR $\alpha$  and RXR $\beta$  in SC-M1 cells and RAR $\beta$  in TSGH9201 cells were defined as 100

Receptor	Cell line				
	SC-M1	TSGH9201	AGS	TMK-1	TMC-1
RAR $\alpha$	100	31	12	20	12
RAR $\beta$	ND	100	ND	18	ND
RAR $\gamma$	100	25	6	19	ND
RXR $\alpha$	100	231	34	35	60
RXR $\beta$	100	219	110	105	39
RXR $\gamma$	ND	ND	ND	ND	ND

ND, not detectable.



**Figure 7.** Expression of RXRs mRNAs in stomach cancer cells. Poly (A)<sup>+</sup> RNA (4 µg per lane) was hybridised with digoxigenin-labelled cDNA for RXR $\alpha$  (a) and RXR $\beta$  (b). Probes were removed and membranes were rehybridised with a  $\beta$ -actin probe as shown on the lower section of each panel.

highest levels of RAR $\alpha$  and RAR $\gamma$  mRNA. TSGH9201 cells also expressed relatively high levels of RAR $\alpha$  and RAR $\gamma$  mRNA compared to tRA-resistant cell lines. RA-resistant cells had markedly decreased levels of the 3.2 kb RAR $\gamma$  mRNA transcript.

Retinoids are differentiation agents. Induction of morphological alterations that are associated with cellular differentiation have been observed in neuroblastoma and acute promyelocytic leukaemia cells treated with retinoids [10, 11]. Our studies show that tRA produced an "enlarged and flattened type" morphology in SC-M1 cells. The morphological alteration is similar to the differentiated epithelial shape observed by Morisaki and associates in gastric cancer cells treated with IL-4 [26]. We did not observe nuclear fragmentation or chromatin condensation, phenotypic changes associated with apoptosis, in SC-M1 cells treated with tRA. Neither did we observe decreased viability of tRA-treated SC-M1 cells. Therefore, our studies suggest that tRA inhibition of SC-M1 and TSGH9201 cell growth is not due to the induction of apoptosis. Further flow cytometric analysis demonstrated the induction of G0/G1 arrest in tRA-sensitive cells. The observation that the growth inhibitory effect of tRA was largely reversed upon its removal from SC-M1 cells supports the suggestion that tRA exerts a cytostatic effect on these cells at concentrations ranging from  $10^{-9}$  to  $10^{-6}$  M. Whether differentiation has occurred during tRA-inhibition of cell growth remains to be confirmed.

The dose-dependent effects of tRA on cell growth were shown in SC-M1 and TSGH9201 cells. Other studies have found that tRA inhibition of cell growth occurs at concentrations ranging

from 1 nM to 10 µM [11–13], similar to the range found to inhibit cell growth in our study. Since the dissociation constant (K<sub>d</sub>) of RAR for tRA is in the nM range [27], the relatively high concentrations of tRA needed for inhibiting cell growth may be due to its instability in culture medium. The highest concentration of tRA achievable in the clinic is approximately  $1 \times 10^{-6}$  M [28, 29]. Therefore, AGS cell growth inhibition by  $5 \times 10^{-6}$  M tRA is probably not clinically relevant. Whether the growth of tRA-sensitive gastric cancer cells, which were inhibited by clinically achievable concentrations of tRA *in vitro*, can be suppressed *in vivo* is worth further investigation.

The levels and expression patterns of RARs mRNA in several types of normal and malignant epithelial cell lines have been examined. Both RAR $\alpha$  and RAR $\gamma$  mRNAs are generally expressed in normal and malignant epithelial cells from the oral cavity, lung and breast [30–32]. However, transformed cells and carcinoma cells frequently lack one or both transcripts of RAR $\beta$  mRNA. Recently, Houle and colleagues suggested that RAR $\beta$  is a tumour suppressor gene and its loss is associated with tumorigenicity *in vivo* [32]. Comparison of RAR $\beta$  expression between normal gastric epithelial and gastric carcinoma cells has not been reported. Whether the absence of expression of RAR $\beta$  transcripts, as observed in SC-M1, AGS and TMC-1 cells in this study, may contribute to neoplastic progression in gastric epithelial cells needs to be further clarified.

The studies presented here demonstrate that tRA, at concentrations up to  $1 \times 10^{-6}$  M, inhibits the growth of SC-M1 and TSGH9201 cells but not AGS, TMK-1 and TMC-1 cells. The mechanism by which tRA regulates gene expression is mediated through the binding of RAR/RXR heterodimers to specific retinoic acid response elements. Therefore, the levels of specific subtypes of nuclear retinoid receptors may influence the sensitivity of cells to tRA. Several studies have shown that RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  play a role in the growth or differentiation of cells from promyelocytic leukaemia [10], epidermoid carcinoma of the lung [32] and teratocarcinoma [33]. Our data show that tRA-sensitive SC-M1 and TSGH9201 cells expressed higher levels of RAR $\alpha$  and RAR $\gamma$  mRNA than did resistant cells. tRA-resistant cells were also found to have markedly decreased levels of the 3.2 kb RAR $\gamma$  transcript (Figure 6). This result suggests that RAR $\gamma$  may play an important role in the growth inhibition of gastric cancer cells by tRA. Since tRA-sensitive SC-M1 cells also expressed high levels of RAR $\alpha$  mRNA, we do not rule out the possible role of RAR $\alpha$  in cellular sensitivity to tRA. Only TSGH9201 and TMK-1 cells expressed detectable levels of RAR $\beta$  mRNA. Previous studies have shown that RAR $\beta$  expression can be stimulated by RA in a cell specific manner [30, 31]. In our studies, RAR $\beta$  mRNA expression was not stimulated by tRA in SC-M1 and AGS cells (S.-Y. Jiang, National Defense Medical Center). This result suggests that RAR $\beta$  may not be related to the growth inhibitory effect of tRA in these cells. RXRs are accessory molecules in the RA mediated pathway, and RXR $\alpha$  is generally expressed in relatively high levels in various cell types. Our data show that the levels of RXR $\beta$  were not correlated with tRA sensitivity of gastric cancer cells. RXR $\beta$  probably does not play a major role in tRA sensitivity in gastric cancer cells. The expression of RXR $\alpha$  in contrast was highest in tRA-sensitive cells. The significance of this observation requires further clarification.

Retinoids are differentiation agents. They have been shown to be effective in the treatment of certain types of cancer and may be useful for cancer prevention. Previous studies have demonstrated a linkage between low serum levels of vitamin A

or  $\beta$ -carotene and the development of gastric cancer as well as the potential for the use of retinoids in the treatment and prevention of gastric cancer. Our studies indicate that gastric cancer cell lines respond differently to tRA *in vitro*, and that different responses may be closely associated to the relative expression of RAR $\gamma$ . Further studies on the mechanisms of growth inhibition *in vitro*, and in nude mice *in vivo* will be important for understanding the potential of retinoids in the chemoprevention or the adjuvant therapy of gastric cancer.

1. Smith MA, Parkison DR, Cheson BD, Friedman MA. Retinoids in cancer therapy. *J Clin Oncol* 1992, 10, 839-864.
2. Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature (London)* 1987, 330, 444-450.
3. Brand NJ, Petkovich M, Krust A, *et al.* Identification of a second human retinoic acid receptor. *Nature (London)* 1988, 332, 850-853.
4. Zelen A, Krust A, Petkovich M, Kastner P, Chambon P. Cloning of murine  $\alpha$  and  $\beta$  retinoic acid receptors and a novel receptor  $\gamma$  predominantly expressed in skin. *Nature (London)* 1989, 339, 714-717.
5. Mangelsdorf DJ, Ong ES, Dyck JA, Evan RM. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature (London)* 1990, 345, 224-229.
6. Hamada K, Gleason SL, Levi BZ, Hirschfeld S, Appella E, Ozato K. H-2RIIP, a member of the nuclear hormone receptor superfamily that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. *Proc Natl Acad Sci USA* 1989, 86, 8289-8293.
7. Mangelsdorf DJ, Borgmeyer U, Heyman RA, *et al.* Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Develop* 1992, 6, 329-344.
8. Heyman RA, Mangelsdorf DJ, Dyck JA, *et al.* 9-cis-retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 1992, 68, 397-406.
9. Gudas LJ. Retinoids, retinoid-responsive genes, cell differentiation and cancer. *Cell Growth Differ* 1992, 3, 655-662.
10. Collins SJ, Bobertson KA, Mueller L. Retinoic acid-induced granulocytic differentiation of HL-60 myeloid leukemia cells is mediated directly through the retinoic acid receptor (RAR- $\alpha$ ). *Mol Cell Biol* 1990, 10, 2154-2163.
11. Sidell N, Altman A, Haussler MR, Seeger RC. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Exp Cell Res* 1983, 148, 21-30.
12. Butler WB, Fontana JA. Responses to retinoic acid of tamoxifen-sensitive and -resistant subline of human breast cancer cell line MCF-7. *Cancer Res* 1992, 52, 6164-6167.
13. Lotan R, Lotan D, Sacks PG. Inhibition of tumour cell growth by retinoids. *Methods Enzymol* 1990, 190, 100-110.
14. Martin S, Bradley J, Cotter T. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin Exp Immunol* 1990, 79, 448-453.
15. Haenszel W, Correa P, Lopez A, *et al.* Serum micronutrient levels in relation to gastric pathology. *Int J Cancer* 1985, 36, 43-48.
16. Miasoedov DV, V'yunitskaia LV, Chernukhina LA, Donchenko GV. Blood vitamin A contents in patients with stomach cancer. *Voprosy Onkologii* 1989, 35, 945-958.
17. Santamaria L, Bianchi A, Ravetto C, Arnaboldi A, Santagati G, Andreoni L. Prevention of gastric cancer induced by N-methyl-N'-nitro-N-nitrosoguanidine in rats fed supplemental carotenoids. *J Nutr Growth Cancer* 1987, 14, 175-181.
18. Fujii T, Yokomori T. A clinical study of vitamin A concerning auxiliary chemotherapies after operation of gastric cancer. *Gan To Kagaku Ryoho* 1991, 18, 265-269.
19. Tzeng C, Meng C, Jin L, Hsieh H. Cytogenetic studies of gastric adenocarcinoma. *Cancer Genet Cytogenet* 1991, 55, 67-71.
20. Ochiai A, Yasui W, Tahara E. Growth promoting effect of gastrin in human gastric carcinoma cell line TMK-1. *Jpn J Cancer Res (Gann)* 1985, 76, 1064-1071.
21. Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 1980, 102, 344-352.
22. Schutte B, Reynders MM, Assche CL van, Hupperets PS, Bosman FT, Blijham GH. An improved method for the immunocytochemical detection of bromodeoxyuridine labeled nuclei using flow cytometry. *Cytometry* 1987, 8, 372-376.
23. Badley JE, Bishop GA, John TS, Frelinger JA. A simple, rapid method for the purification of poly A<sup>+</sup> RNA. *Biotechniques* 1988, 6, 114-116.
24. Fyrberg EA, Kindle KL, Davidson N. The actin genes of drosophila: a dispersed multigene family. *Cell* 1980, 19, 365-378.
25. Zhou L, Pang J, Munroe DG, Lau C. A human retinoic acid receptor gamma isoform is homologous to the murine retinoic acid receptor gamma 7. *Nucleic Acid Res* 1993, 21, 520.
26. Morisaki T, Yuzuki DH, Lin RT, Foshag LJ, Morton DL, Hoon DSB. Interleukin 4 receptor expression and growth inhibition of gastric carcinoma cells by interleukin 4. *Cancer Res* 1992, 52, 6059-6065.
27. Cretaz M, Baron A, Siegenthaler G, Hunziker W. Ligand specificities of recombinant retinoic acid receptors RAR $\alpha$  and RAR $\beta$ . *Biochem J* 1990, 272, 391-397.
28. Lee JS, Newman RA, Lippman SM, *et al.* Phase I evaluation of all-trans-retinoic acid in adults with solid tumors. *J Clin Oncol* 1993, 11, 959-966.
29. Smith MA, Adamson PC, Balis FM, *et al.* Phase I and pharmacokinetic evaluation of all-trans-retinoic acid in pediatric patients with cancer. *J Clin Oncol* 1992, 10, 1666-1673.
30. Gaetano C, Matsumoto K, Thiele CJ. Retinoic acid negatively regulates p34<sup>cdc2</sup> expression during human neuroblastoma differentiation. *Cell Growth Differ* 1991, 2, 487-493.
31. Hu L, Crowe DL, Rheinwald JG, Chambon P, Gudas LJ. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res* 1991, 51, 3972-3981.
32. Houle B, Rochette-Egly C, Bradley WEC. Tumor-suppressive effect of the retinoic acid receptor  $\beta$  in human epidermoid lung cancer cells. *Proc Natl Acad Sci USA* 1993, 90, 985-989.
33. Moasser MM, DeBlasio A, Dmitrovsky E. Response and resistance to retinoic acid are mediated through the retinoic acid receptor  $\gamma$  in human teratocarcinomas. *Oncogene* 1994, 9, 833-840.

**Acknowledgements**—The authors thank Dr C.-L. Meng for providing SC-M1 cells, Dr E. Tahara for providing TMK-1 cells, Dr P. Chambon for providing cDNA probes for mouse RAR $\alpha$  and human RAR $\gamma$ , Dr M. Pfahl for providing the cDNA probe for human RAR $\beta$ , Dr R.M. Evans for providing cDNA probes for human RXR $\alpha$  and mouse RAR $\gamma$ , Dr K. Ozato for providing a cDNA probe for human RXR $\beta$ , Dr W.F. Dove for providing a cDNA probe for *Drosophila* actin, Ms Lily Chao and Hsiang-Ning Hsiao for technical assistance.