Eur J Cancer, Vol. 29A, Suppl. 5, pp. S9–S13, 1993.
Printed in Great Britain

0959–8049/93 $6.00 + 0.00

Retinoic Acid and Interferon Combination Studies in Human Cancer

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Retinoic acid and interferon-α have limited single-agent activity in advanced cancer. Cell culture data indicate that in combination these agents have enhanced activity (modulating growth and differentiation) in a number of malignant cell types. Recent clinical work in advanced squamous cell carcinoma reports major activity with this regimen. This paper reviews the preclinical and clinical data testing retinoic acid in combination with interferons and presents recent work integrating these agents with radiotherapy in locally advanced cervical cancer.
Eur J Cancer, Vol. 29A, Suppl.5, pp. S9–S13, 1993.

INTRODUCTION

BOTH INTERFERONS (IFNs) and retinoids are known to possess antiproliferative, differentiative, and immunomodulatory properties [1, 2], but they probably exert these effects through separate molecular mechanisms. Thus, the potential exists for complementation of retinoid activity by IFNs and vice versa, just as combining cancer chemotherapeutic agents with different mechanisms of cytotoxicity frequently results in additive and sometimes synergistic cell killing. A growing body of evidence from both laboratory and clinical research now supports the concept that simultaneous exposure to both retinoids and IFNs can result in enhanced antiproliferative and differentiative effects compared with either single agent alone. Further, there is intriguing data that suggests that these agents may interact at the molecular level to produce synergistic effects.

Preclinical retinoic acid – interferon interaction data

Much of the work in this area has been performed in myeloid leukaemia cells, especially the human cell line, HL-60. Grant *et al* [3] made the first critical observation regarding the interaction of these compounds in a subline of HL-60 which was resistant to retinoid-induced differentiation and antiproliferative effects. Recombinant IFN-αA (1000 U/ml) and β-all *trans* retinoic acid (TRA) (50 μmol/l), when used as single agents, resulted in minimal growth inhibition, approximately 80% of control, in the retinoic acid-resistant cells. However, combined exposure to IFN-αA and TRA reduced growth in suspension to less than 10% of control levels. This potentiation of antiproliferative effect by combined treatment was also observed when colony formation in soft agar was studied. IFN-αA alone did not induce differentiation in the parent HL-60 cells or the retinoic acid-resistant subline, assessed by the ability of treated cells to reduce nitroblue tetrazolium (NBT). However, addition of IFN-αA to concentrations of TRA ranging from 10 to 50 μmol/l resulted in a 3 to 20-fold enhancement of NBT reduction in the resistant cells. A similar effect on differentiation was observed in the parental cells, though to a smaller extent (2-fold enhancement).

Hemmi and Breitman [4] next studied the ability of various IFNs, γ, -αA, and -αD, in combination with TRA (0.01 μmol/l), to induce differentiation of HL-60 cells to morphologically and functionally mature monocyte-like cells. None of the IFNs alone (1000–5000 U/ml) substantially inhibited growth or induced markers of myelomonocytic differentiation, including NBT reduction, non-specific esterase and 5′-nucleotidase activities after 96 h of exposure. Similarly, a low concentration of TRA (0.01 μmol/l) resulted in only a marginal increase in NBT reduction (23%). Combined treatment with TRA and IFN-γ (1000 U/ml) resulted in greater than additive effects on all markers of myelomonocytic differentiation, including morphology (82% of population mature monocytes). Although both IFN-αA and -αD also potentiated TRA effect they were both far less potent than IFN-γ.

Using only NBT reduction to assess differentiation, Peck and Bollag [5] evaluated a variety of retinoids (10 μmol/l) alone and in combination with IFNs— α (1000 U/ml), β (1000 U/ml), and γ (100 U/ml) in both HL-60 cells and U937 cells, a human histiocytic lymphoma line. As found in the previous studies, IFNs alone were not capable of inducing differentiation of either line. These authors identified Ro-13-6307, TRA, and 13-*cis*-retinoic acid (13cRA) as the most active retinoids, both alone and in combination with IFNs. They also found that IFN-γ markedly potentiated retinoid effects, especially with TRA, and was significantly more potent than the α and β proteins. Of interest, other cytokines including tumour necrosis factor-α, interleukins, and granulocyte-colony stimulating factor, also possessed the ability to enhance the differentiation-inducing effects of retinoids in these particular cell lines. However, combinations of multiple cytokines with retinoids frequently resulted in antagonistic effects.

The interaction of retinoids and IFNs in primary cultures of human acute myeloid leukemia cells has been reported by two groups. Gallagher *et al.* [6] evaluated the effect of TRA and IFN-αA, both singly and in combination, on growth and morphologi-

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cal differentiation in leukaemic blasts from 10 patients. Primary cultures from these patients were generally resistant to IFN-αA, with only 2/10 exhibiting growth inhibition at doses less than 2500 U/ml. Similarly, 4 other cases were the only ones sensitive to growth inhibition by TRA (0.05 – 0.5 μmol/l). Antiproliferative effects greater than single agent activity alone could be demonstrated in primary culture for only 3 cases, 2 of which were FAB classification 3 (APL), and sensitive to TRA. These 3 cases and one other showed marginally increased morphological differentiation during combined treatment, as opposed to IFN-αA or TRA alone. However, a greater than additive effect was demonstrated in only one. More recently, Nakamaki *et al.* [7] found that TRA (0.01 μmol/l) induced granulocytic or monocytic differentiation in 5/8 cases (FAB classification M1, 3 and 4). IFN-α and -γ (1000 and 100 U/ml, respectively) demonstrated minimal activity alone, but appeared to potentiate the effects of TRA in the 5 cases which were sensitive to TRA alone. In contrast to the findings of Grant *et al.* [3] discussed above, IFNs could not overcome TRA resistance. As previously reported by others, IFN-α tended to induce differentiation along granulocytic, and IFN-γ along monocytic pathways. In one case, combination treatment altered granulocytic differentiation induced by TRA alone to monocytic in the presence of IFN-γ.

The effect of combining retinoids and IFNs has also been examined in human solid tumour lines, but with the exception of neuroblastoma, data are available only for antiproliferative properties. Marth *et al.* [8] have reported greater than additive effects on growth inhibition when four human breast carcinoma lines were exposed to TRA (1 μmol/l) and IFN-γ (50 – 500 U/ml). Of note, this cooperativeness was seen in cell lines resistant to either agent alone. IFN-α, in contrast, resulted in only additive or less than additive effects, in combination with TRA, in three of the four lines. A human non-small cell lung cancer and laryngeal cancer line were also studied; however, neither of these lines exhibited greater than additive effects with combinations of TRA and IFN-α or γ.

Frey *et al.* [9] compared the activity of TRA, 13cRA, and acitretin with and without IFN-α on growth inhibition in HL-60, MCF7 (breast carcinoma), and three squamous cell carcinomas (SCC4 and SCC15 tongue, and A431, vulva). These solid tumour cell lines were generally resistant to retinoids compared to HL-60, requiring 3 – 30 μmol/l concentrations for significant (≥ 30% inhibition) effects, with only minimal differences observed among the three compounds. Conversely, all of the carcinoma lines, with the exception of A431, were more sensitive to IFN-α (1000 U/ml) than HL-60. Maintaining a constant dose of IFN-α, these investigators confirmed potentiation of antiproliferative effects with all three retinoids in a dose range from 0.03 to 30 μmol/l in HL-60 and MCF7 cells. However, results of combination treatment were less profound in the other three carcinoma lines, with greater than additive effects seen consistently only in SCC4 and only with the two highest doses of retinoids, 3 and 30 μmol/l.

In a recent paper, Higuchi *et al.* [10] utilised a neuroblastoma cell line, NUB-6, which undergoes differentiation, as assessed by formation of neurite-like processes, under the influence of TRA (1 μmol/l) or IFNα2 (1000 U/ml). Combined treatment with these same doses results in an approximate 4-fold enhancement of differentiation effect.

Although many of the *in vitro* studies summarised above are marred by the use of supraphamacological doses of both retinoids and IFNs and often with continuous exposure for prolonged periods of time, they do document intriguing interactions between these compounds which have stimulated new strategies in the clinic.

POTENTIAL MOLECULAR MECHANISM(S) OF RETINOIC ACID–INTERFERON INTERACTION

Many of the effects of retinoids on cell growth and differentiation are thought to be the direct or indirect result of changes in gene expression. The mechanisms by which retinoids affect gene expression are beginning to be unravelled. A major advance towards elucidation of these mechanisms was the discovery of nuclear retinoic acid receptors (RARs), which exhibit DNA sequence homology to the family of steroid hormone receptors, particularly in the DNA-binding domain [11–14]. The similarity of the RARs to steroid hormone receptors suggests that they also act as ligand-activated DNA-binding proteins that regulate gene transcription via interaction with responsive elements in the promoter region of specific genes. Three RAR subtypes designated RAR-α, RAR-β and RAR-γ were cloned from human and mouse cell lines [11, 12, 14–19]. It has been suggested that the different receptor subtypes have distinct functions because they differ in tissue distributions in adult tissues, as well as in patterns of expression in the developing embryo [12, 20–24]. It is noteworthy that the affinities of different retinoids for binding RARs differ significantly. For example, the affinity of RAR-α for TRA is about 5-fold higher than for 13cRA [25]. More recently, another RA receptor family (RXR), which is vastly different in DNA sequence from the RARs and exhibits a considerably lower affinity for RA was discovered [26]. It was suggested that this receptor represents an evolutionarily distinct retinoid response pathway from that of the RARs.

It seems intuitively likely that RARs are the ultimate mediators of the action of retinoids on gene expression. However, direct proof for that conclusion is available for only a few cases. RARs were identified in HL-60 leukaemia cells, which are induced to undergo myeloid differentiation by TRA [27, 28]. An RA-resistant mutant subclone of HL-60 cells was found to contain a defective RAR-α, which apparently was the cause for resistance to TRA because transduction of a single copy of RAR-α into the mutant cells restored responsiveness to TRA [29]. Furthermore, TRA unresponsive K-562 cells, derived from a patient with chronic myelogenous leukaemia at the blast crisis stage, were found to express a very low level of RAR-α compared with HL-60 cells. Retroviral-mediated transduction of RAR-α cDNA into the K562 cells, which resulted in an increase in the number of receptors, rendered the cells responsive to TRA [30]. TRA treatment of the transfected cells resulted in a decrease in cell proliferation but there was no evidence for TRA-induced differentiation, suggesting that the increased level of RAR-α receptors can mediate RA effects on proliferation but not necessarily on the differentiation of these cells [30].

Although the above results strongly support a direct role for at least RAR-α in mediating RA actions, the presence of RARs may not be sufficient to render a cell responsive to the growth inhibitory or differentiation-inducing effects of retinoids. For example, RAR-α is expressed by most human leukaemias (fresh cells and cell lines) regardless of their responsiveness to RA [31–33]. Other proteins including nuclear transcription factors (e.g. c-*jun* [34] and the RAR co-regulatory proteins [35]), may be required for the formation of a transcriptionally competent complex among RAR, polymerase and DNA.

The synergistic activities of combinations of retinoids and interferons may be the result of different mechanisms. One possibility is that retinoids increase the expression of IFN

receptors. This possibility has been excluded in the case of breast carcinoma BT20 cells by Marth *et al.* [36] who found no increase in IFN-γ receptor level after TRA treatment. However, TRA enhanced the down regulation of IFN-γ receptor by IFN-γ. Further studies by this group demonstrated that IFN-γ had no effect on the level of the cytoplasmic retinoic acid binding protein in BT-20 and ZR-75-1 breast carcinoma cells [36]. The possibility that IFNs induce or increase the expression of RARs and thereby render cells more sensitive to the action of retinoids has not been investigated and merits examination. IFNs could also induce the expression of co-regulatory nuclear proteins that interact with RARs and thereby influence affinity for binding response elements in different genes [35].

Another possible mechanism of the combined actions of retinoids and IFNs was provided by several studies, which found that TRA enhanced the induction of 2′-5′-oligoadenylate synthetase by IFN-γ in human histiocytic lymphoma U937 and WISH cells [37] and in human breast carcinoma cells BT-20 [36]. Higuchi *et al.* [10], who found that TRA acted post-transcriptionally to stabilise the 2′-5′-oligoadenylate synthetase mRNA, provided a more concrete basis for this combination. That this effect of TRA is specific for the differentiative effect of IFN was suggested by the finding that TRA failed to enhance 2′-5′-oligoadenylate synthetase level in GOTO neuroblastoma cells which are growth inhibited but are not induced to differentiate by IFN-α2 [10].

Since retinoids and IFNs alter the expression of growth factors (e.g. TGF-β and EGF-R), affect angiogenesis, programmed cell death, and augment immune responses, they may act additively or synergistically via these mechanisms.

CLINICAL COMBINED RETINOIC ACID–INTERFERON-α STUDIES

Single-agent therapy with retinoic acid or interferon-α has limited activity in therapy of advanced solid tumours with major activity limited to TRA in acute promyelocytic leukaemia and IFN-α in hairy cell leukaemia [38, 39]. The major single-agent activity has been reported in premalignant disorders and minimal burden cancer (e.g. chronic phase myelogenous leukaemia, adjuvant therapy).

The rationale for combined retinoic acid and interferon-α in cancer therapy is strong and stems from preclinical studies showing their differing mechanisms of action and *in vitro* interaction. Further support for the combination is in the non-overlapping and reversible toxicities. Retinoic acid causes mucocutaneous dryness and IFN-α causes a flu-like syndrome and fatigue.

Trial in squamous cell carcinoma of the skin

The rationale for the study of this combination for advanced squamous cell carcinoma (SCC) of the skin is supported by the activity of both agents in reversing skin premalignancy and the activity of retinoic acid in preventing invasive skin cancer in high-risk xeroderma pigmentosum patients [1, 40]. Single-agent systemic therapy with retinoic acid or IFN-α has produced a response rate of 40 – 50% in locally-advanced SCC of the skin. The complete response rate was less than 20%. The single-agent data were based on a small series of high-dose trials.

A clinical trial of 13cRA and IFN-α2a has been recently conducted based on the general rationale for the combination described earlier, the single-agent *in vivo* activity and enhanced *in vitro* inhibitory effects in SCC cell culture [41]. This is the only reported phase II trial of any systemic therapy (including cytotoxic) in cutaneous SCC. The overall response rate was 68% in 28 patients with a complete response in 7 of the 19 responding patients. The response rate appeared to be related to disease extent. Major responses occurred in 13 of 14 patients with locally advanced disease, 4 of 6 regionally advanced patients and 2 of 8 with distant metastatic disease. This trend of lower response rates with increased disease extent is statistically significant. Toxicities were characteristic of single agent side-effects. The major limiting toxicity in this elderly group (median age > 65 years) was fatigue.

These phase II results (> 80% response rate in locally and regionally advanced disease) compare favourably to (a) the 60 – 70% response rate in small series of multi-agent platinum and non-platinum containing regimens in a similar locally advanced patient population and (b) the single agent biological data with higher doses in locally advanced disease—suggesting supra-additive *in vivo* activity.

Based on the positive data in the pilot trial of advanced SCC of the skin, a series of phase II trials were developed in advanced SCC of the cervix, head and neck, lung and melamona. No major activity was observed in recurrent head and neck cancer SCC [42] or in metastatic lung SCC [43] or metastatic melanoma [44]. The phase II trial in locally advanced cervical cancer indicates major activity of combined 13-cRA-IFN-α2a and the clinical data will be briefly summarised.

Cervical cancer studies

Cervical cancer is a leading cause of cancer death in developing countries. Standard therapy for locally advanced disease is irradiation which produces 5-year long-term survival in less than 50% of treated patients [45, 46]. Chemotherapy has not improved survival in any disease setting [45]. In advanced disease, limited numbers of single agents have produced response rates in the range of 15 to 35%. Multi-agent cytotoxic regimens in the neoadjuvant setting have produced overall response rates of 40 – 60%, but have not produced improved survival in phase III radiotherapy-controlled trials [47, 48]. Concurrent multi-agent cytotoxic radiotherapy regimens are limited by myelosuppression. Concurrent single-agent cytotoxic radiotherapy regimens are under active study with mixed results [49–51]. Metastatic disease is incurable. A major research focus in cervical cancer is the development of new active agents and agents to potentiate radiotherapy effects (radiosensitisers) in locally advanced disease [45].

Based on the *in vivo* SCC of the skin data, preclinical data on the interaction of interferon-retinoic acid, the different toxicity profiles and the antiviral activity of these agents (Table 1), we developed a phase II trial in locally advanced cervical carcinoma at the Instituto Jaliscience de Cancerologia Guadalajara, Mexico [52–55]. In Mexico, as in most developing countries, cervical cancer presents in symptomatic late stages and is the number one cause of cancer death in women, *overall* cancer morbidity and *overall* cancer mortality between the age of 24 and 65 years.

The dose of the retinoic acid in the cervical cancer trial remained the same and the interferon dosage was increased from 3 to 6 mU per day in this younger patient population. The initial report of 26 patients with limited follow-up described major clinical activity [56]. This trial has recently been completed with 32 registered patients — all evaluable for response and toxicity [57]. Sixteen, or 50% had major tumour regression (greater than 50% reduction in bidimensional measurements), 4 patients had a complete response. The major response rates are roughly 50% in patients with (a) locally advanced, *bulky* (at least one dimension greater than or equal to 10 cm) FIGO stage II B, III

B, and IV (Mo) disease and (b) *poorly differentiated* tumours. At a median follow-up of 18 months, the median survival has not been reached. Of the 16 responders, 9 relapsed with a median response duration of three months. The time to major response (≥ PR) was ≤ 2 months in 15 of the 16 responders. There was no effect of 25% dose escalation (of both agents) in non-responders. Study compliance was excellent and drug toxicity was minimal in this younger group of patients (median age 42 years).

Current work in locally advanced disease includes the integration of irradiation. As stated above, radiotherapy, standard for locally advanced cervical cancer, achieves < 50% cure rates and neoadjuvant chemotherapy has no beneficial survival impact [42–46]. Concurrent chemoradiotherapy is under active study with mixed results. Current U.S. phase III studies include the inactive agent hydroxyurea (as a potential pure radiosensitiser). The published data suggest an altered pattern and timing of disease failure, but no clear survival improvement [47–49]. The dose-limiting toxicities of concurrent hydroxyurea radiotherapy are cystitis and myelosuppression.

The rationale for adding RA-IFN-α to standard radiotherapy is based on the independent major activity of this biological agent combination *and* the reported additive and synergistic effects of these agents and radiation in mammalian cells in tissue culture [55–57].

The current study consists of two phases: a 2-month full dose induction (based on the time to major response data from the first study) followed by a 2-month phase of concurrent radiotherapy plus modified dose 13-cRA-IFN-α. The preliminary data from this ongoing study are briefly summarised here. The 2-month induction response rate of 42% (10/24) [95% C.I. 22 – 61%] is consistent with the initial full phase II trial response rate of 50% (95% C.I. 33 – 67%). Multiagent cisplatin-based chemotherapy in the neoadjuvant setting produces 40 – 60% response rates after two to four cycles in stage IIB – IIIB disease [45, 46], similar to the 13-cRA-IFN-α data from two studies [54, 58]. The second phase is ongoing and the optimal dose and schedule of 13-cRA-IFN-α added to comprehensive radiotherapy (external and intracavitary) is not yet determined. The dose-limiting toxicity of the concurrent phase is radiation proctitis.

SUMMARY

Active biological regimens offer new combined modality options for primary, adjuvant and salvage therapy of cancer. This paper reviewed one such promising combination — retinoic acid plus IFN-α. Based on these data, further retinoic acid–IFN clinical trials are indicated. Recent work has now indicated substantial activity in advanced SCC of the skin and cervix. Current work is studying the complex interaction and integration of 13cRA, IFN-α and radiation in primary therapy of locally advanced cervical cancer. Further work will focus on the integration of cytotoxics and other cytokines directed by preclinical (*in vitro* and *in vivo*) data and on biological studies using laboratory endpoints as indicators of early (subclinical) activity.

Table 1. *13-cRA + IFN-α in cervical cancer rationale*

Laboratory
Differentiative, proliferative, immunomodulatory, antiviral, anti-angiogenic activity
Different mechanisms (receptors)
Combined activity *in vitro* (leukaemia and solid tumours) and *in vivo* (solid tumours)
Clinical
Single-agent data: active in preinvasive cervical disease (no major activity in advanced disease)
Non-overlapping toxicity
High activity in advanced SCC of the skin*

* *JNCI* 1992, **84**, 235–241.

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Acknowledgements—This study was supported in part by a grant from F. Hoffmann-LaRoche Ltd. Dr. Lippman is a recipient of a Career Development Award from the American Cancer Society.