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PROSPECTS

Experimental Basis of Cancer Combination Chemotherapy With Retinoids, Cytokines, 1,25-Dihydroxyvitamin D_3, and Analogs

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Abstract Retinoids, cytokines as well as 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and analogs possess properties known to contribute potentially to cancer chemopreventive and chemotherapeutic effects. They induce cell differentiation, inhibit cell proliferation, suppress expression of viral oncogenes, and inhibit angiogenesis necessary for tumor growth. Since clinical combination chemotherapy of cytotoxic agents has proven superior to monotherapy, this modality might also be useful for other classes of antitumor drugs. A series of retinoids, such as all-trans-, 13-cis-, 9-cis retinoic acid, and acitretin, cytokines, 1,25$(OH)_2D_3$, and analogs have been investigated in model systems of differentiation, proliferation, viral oncogenes, and angiogenesis. The three classes of compounds have common effects but nevertheless show a variance depending on the particular representative of each class. Combination of compounds of the different classes led in the various models to a higher efficacy compared with the compounds given alone. Cytokines such as IFNα, IFNγ, G-CSF, TNFα, IL-1, and IL-4 markedly potentiate the differentiation-inducing effect of retinoids. Cytokines as well as retinoids combined with 1,25$(OH)_2D_3$ and analogs synergistically enhanced differentiation induction in human transformed hemopoietic cell lines. On a series of human transformed epithelial cell lines a panel of cytokines, such as IFNα, IFNγ, TNFα, TGFβ, and EGF acted synergistically with retinoids on inhibition of proliferation. This was also observed by combining retinoids with 1,25$(OH)_2D_3$ and analogs. Retinoids as well as interferons α and γ have the capacity to suppress the oncogene expression of human papilloma viruses which are involved in induction and growth of certain malignancies such as cervical cancer. All-trans-, 13-cis-, 9-cis retinoic acid, and acitretin as well as IFNα inhibited the formation of newly formed blood vessels induced by injection of HPV harboring, tumorigenic cell lines into Balb/C mice. The combination of retinoids with IFNα was more efficacious in inhibiting angiogenesis than a retinoid or IFNα given alone. Since there is evidence that cell differentiation, cell proliferation, viral oncogene expression as well as angiogenesis play a role in tumor induction and/or progression of tumor growth, retinoids, cytokines, as well as 1,25$(OH)_2D_3$ and analogs may be useful in chemoprevention and chemotherapy of neoplasms. Based on the superior effect of combinations compared with administration of single compounds of these three classes under experimental conditions there is hope that also clinical application of combination treatment might bring us a step forward in chemoprevention and chemotherapy of cancer. This has already been proven in a very limited number of clinical trials.

Key words: combination chemotherapy, retinoids, cytokines, vitamin D, human transformed cell lines

During the period between 1945 and 1970 a large series of cytotoxic agents have been found which enabled oncologists to add to their therapeutic armamentarium of surgery and radiotherapy a third possibility, that of tumor chemotherapy. In the first era these chemotherapeutic agents have been administered alone and sequentially. A marked progress was achieved when Frei and collaborators from the Acute Leukemia Group B [Frei et al., 1961] reported in 1961 that simultaneous application of methotrexate and mercaptopurine to patients with acute lymphocytic leukemia led to a higher remission rate and a longer survival time than sequential therapy with the single agents. In 1970 De Vita and collaborators could demonstrate a high remission and cure rate in patients with advanced Hodgkin's disease treated by combination of four different chemotherapeutic agents. These discoveries were the starting point for numerous clinical trials with combination of cytotoxic agents producing in many neoplastic diseases,

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such as leukemias, lymphomas, teratocarcinomas, mammary carcinomas, lung carcinomas, etc., superior results compared to therapy with single agents. In the last few years new classes of compounds such as retinoids and cytokines, not belonging to cytotoxic agents have found a place in the treatment of a limited number of oncological indications.

This article will concentrate on laboratory experiments obtained with retinoids, cytokines, $1,25(OH)_2D_3$, and analogs. Like in the case of cytotoxic agents the combination of the above-mentioned classes of compounds produces an enhanced antitumor activity in preclinical experiments. First, clinical therapeutic results with such combination therapies have been reported [Lippman et al., 1992a,b] and rise hope for further progress in this domain. The experimental model systems on which a predictive value might be based concern models on cell differentiation, cell proliferation, viral oncogene expression, and angiogenesis.

CELL DIFFERENTIATION

Induction of differentiation in human transformed hemopoietic cell lines has been demonstrated already in the late seventies [Breitman et al., 1983]. However only recently differentiation therapy has been realised in the successful clinical therapy of acute promyelocytic leukemia with a very high rate of complete remissions [Huang et al., 1988; Degos et al., 1990; Warrell et al., 1991; Ohno et al., 1991; Chen et al., 1991]. A series of retinoids has been investigated for their differentiation inducing effect particularly in the acute myelocytic leukemia cell line HL-60. This effect was measured by determining the nitroblue tetrazolium (NBT) reduction, characteristic for the differentiated granulocytes. When retinoids were given alone, 9-cis retinoic acid (9-cis RA) was most efficacious in inducing differentiation followed by all-trans retinoic acid (all-trans RA), 13-cis retinoic acid (13-cis RA), and the aromatic retinoid acitretin [Bollag and Peck, 1992; Bollag and Holdener, 1992; Peck and Bollag, 1991; Brockhaus and Bollag, unpublished] (Fig. 1). Since clinical application of retinoids is still limited to a few oncological indications there is a need to improve differentiation therapy by combining retinoids with other classes of compounds. A panel of cytokines has been investigated in spite of the fact that in the HL-60 line when given alone they did not exert a differentiating effect. However, in combination

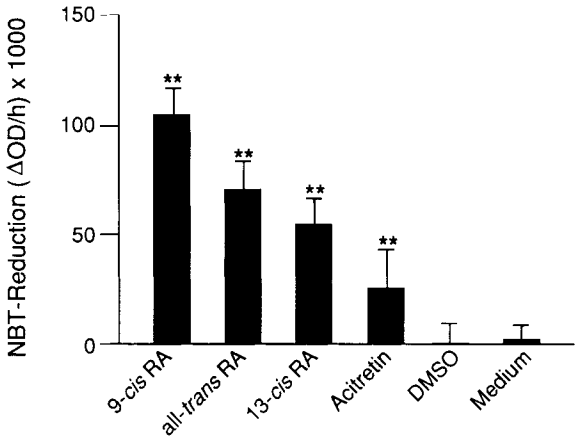

Fig. 1. Induction of differentiation in HL-60 cells by various retinoids (10^{-6} M). $^{**}P < 0.005$ compared with DMSO control (Student's *t*-test).

they potentiated substantially the differentiation inducing effect of the retinoids [Bollag and Peck, 1991; Bollag and Holdener, 1992; Peck and Bollag, 1991]. The highest degree of differentiation was achieved by the combination with IFNγ and G-CSF, but also IFNα, IFNβ, TNFα, IL-1, and IL-4 were active to a lower degree (Fig. 2). Rather similar results were accomplished by using the myelomonocytic cell line U937. $1,25(OH)_2D_3$, the hormonally active form of vitamin D, when administered alone induces differentiation in transformed hemopoietic cell lines, such as HL-60 and U937, even in lower molar concentrations than the retinoids [Imai et al., 1992; McCarthy et al., 1983; Murao et al., 1983; Olsson et al., 1983]. Nevertheless, this compound was not active in clinical differentiation therapy probably because the high doses necessary for a successful therapy could not be given because of increased intestinal calcium absorption, bone calcium mobilisation, hypercalcemia and calcium tissue deposition. A series of analogs of $1,25(OH)_2D_3$ with markedly less influence on calcium metabolism but still exerting a strong differentiation-inducing effect on HL-60 and U937 cells have been found, such as $1,25(OH)_2$-16-ene-23-yne D_3 [Norman et al., 1990; Zhou et al., 1989], 20-epi-$1,25$-$(OH)_2D_3$ [Lee et al., 1992] or calcipotriol [Binderup and Bramm, 1988]. Combination of retinoids with $1,25(OH)_2D_3$ [Gullberg et al., 1985; Miyaura et al., 1985] or its analogs [Doré et al., 1992], particularly those being less handicapped by calcium liabilities is a promising approach for improving differentiation therapy. In HL-60 cells 9-cis RA was most active when combined with

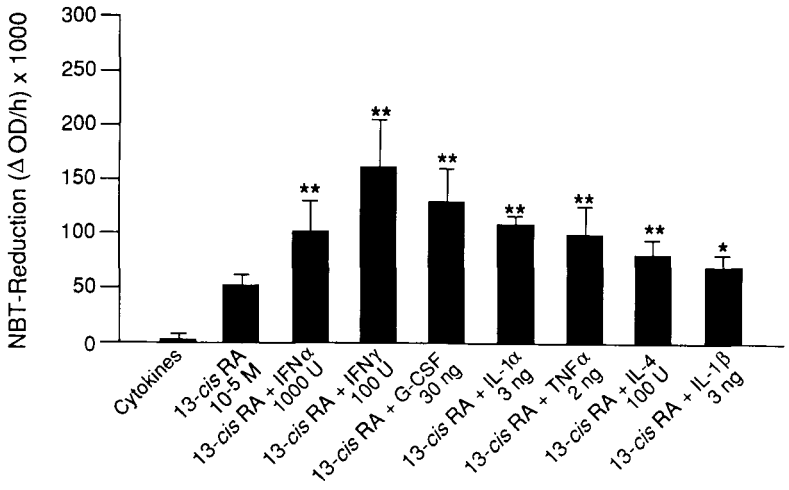

Fig. 2. Induction of differentiation in HL-60 cells by 13-cis RA or cytokines alone and in combination. Concentrations of cytokines are given per ml. $^*P < 0.05$, $^{**}P > 0.005$ (Student's *t*-test). Reproduced from Bollag and Peck [1991] with permission of the publisher, Karger, Basel, Switzerland.

$1,25(OH)_2D_3$ followed by all-trans RA and 13-cis RA leading in all cases to a higher differentiating effect than when the single compounds were administered alone [Brockhaus and Bollag, unpublished] (Fig. 3). In addition, by the combination of $1,25(OH)_2D_3$ or its analogs with cytokines such as IFNα, IFNβ, IFNγ, TNFα, IL-1, and GM-CSF a synergism in differentiation induction of transformed hemopoietic cell lines such as U937 and M1 was observed [Petrini et al., 1991; Gullberg et al., 1985; Kelsey et al., 1990; Zuckerman et al., 1988; Nakaya et al., 1991].

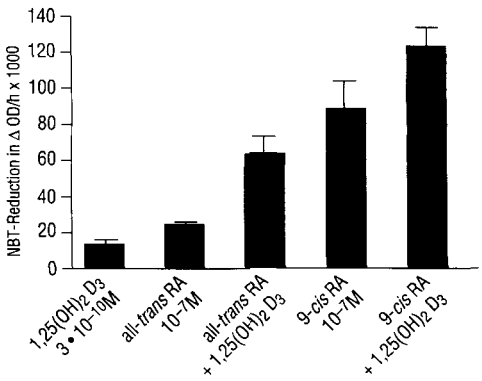

Fig. 3. Induction of differentiation in HL-60 cells by all-trans RA and 9-cis RA (10^{-7} M), $1,25(OH)_2D_3$ (3×10^{-10} M) and their combination. $P < 0.005$ when combinations were compared with RAs and $1,25(OH)_2D_3$ given alone (Welch-Aspin-test).

CELL PROLIFERATION

Besides induction of cell differentiation the antiproliferative action undoubtedly contributes to a major part to the antitumor activity not only of cytostatic agents but also of retinoids, cytokines, $1,25(OH)_2D_3$, and analogs. The antiproliferative effect of all-trans RA was demonstrated in a series of different human transformed cell lines of hemopoietic and epithelial origin [Lotan, 1980; Ponec et al., 1988; Jetten et al., 1990; Sacks et al., 1989]. Four human carcinoma cell lines, MCF7 mammary carcinoma, SCC4 and SCC15 squamous cell carcinomas of the tongue and A431 squamous cell carcinoma of the vulva were used to test inhibition of proliferation by various retinoids [Frey et al., 1991; Bollag et al., 1992]. Proliferation rates were determined by the capacity of viable cells to reduce MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], an indicator of mitochondrial enzyme activity. All-trans RA, 13-cis RA, and acitretin inhibited the proliferation of the four human transformed epithelial cell lines. Although each cell line had its own profile of proliferation inhibition by retinoids, dependent on the cell line tested, the difference in response to the various retinoids was not very marked. In a second experiment the three isomers all-trans RA, 13-cis RA, and 9-cis RA were compared with regard to inhibition of proliferation in SCC15 and A431 cells (unpublished). Effective growth inhibition was observed already at 3×10^{-8} M concentration. All-trans, 13-cis, and 9-cis RA exerted a rather similar antiproliferative effect. This was in contrast to the clearly varying effect of the three retinoic

acid isomers on induction of differentiation. A series of cytokines were examined for their growth modulating effect given either alone or in association with retinoids. The following cytokines had an antiproliferative effect on MCF7, SCC4, SCC15, and A431 cell lines: IFNα, IFNγ, TNFα, TGFβ, and EGF. Depending on the cell line tested their growth inhibiting effect varied markedly, whereby IFNα had the most consistent influence [Bollag and Peck, 1993]. Combination of the retinoids with the mentioned cytokines increased substantially the antiproliferative effect. The synergism in the combination experiments with the tested cell lines did hardly vary depending on the different retinoids, all-trans RA, 13-cis RA, 9-cis RA, and acitretin, but was clearly dependent on the cytokine administered [Bollag et al., 1992; Bollag and Peck, 1993; unpublished]. MCF7 cell proliferation was most markedly inhibited by TGFβ and TNFα and to a lesser degree by IFNα followed by IFNγ. IFNα elicited the most marked synergism with retinoids in SCC4 cells (Fig. 4), followed in descending order of activity by EGF, TGFβ, and IFNγ. IFNα was the only cytokine which showed synergistic activity in combination with retinoids against SCC15 cells (Fig. 5), whereas in A431 cells EGF elicited the strongest synergistic response followed by IL-1, IFNα, and IFNγ. Thus every cell line had a unique profile of proliferation inhibition by a particular cytokine when combined with retinoids. $1,25(OH)_2D_3$ has been demonstrated to inhibit proliferation in a series of human transformed cell lines such as the mammary carcinoma cells MCF7, T47D (estrogen receptor positive) and BT20 (estrogen receptor negative), the colonic carcinoma cells CaCo-2, and the melanoma cells MM6 [Chouvet et al., 1986; Cross et al., 1992; Frampton et al., 1983; Eisman et al., 1989]. Also $1,25(OH)_2D_3$ analogs affecting the calcium metabolism to a lower degree than $1,25(OH)_2D_3$ itself, e.g., calcipotriol [Colston et al., 1992], EB 1089 [James et al., 1992], 1,25-$(OH)_2$-16-ene-23-yne D_3 [Cross et al., 1993], and 22-oxa-$1,25(OH)_2D_3$ [Abe et al., 1991] inhibited proliferation of various mammary carcinoma cell lines and a colonic carcinoma cell line. With a combination of all-trans RA and $1,25(OH)_2D_3$ a synergistic growth inhibitory effect was achieved in the human breast cancer cell line T47D [Koga and Sutherland, 1991]. It might be also of interest to combine retinoids and $1,25(OH)_2D_3$ or analogs in animal experiments with xenografts or chemically induced tumors since retinoids as well as $1,25(OH)_2D_3$ and analogs have proven to exert a preventive as well as a therapeutic activity on these tumors [Colston et al., 1992; Abe et al., 1991; Bollag and Hartman, 1983; Eisman et al., 1987; Smith et al., 1993]. In chemically-induced mammary cancer further investigations with a combination of these agents together with tamoxifen may be promising.

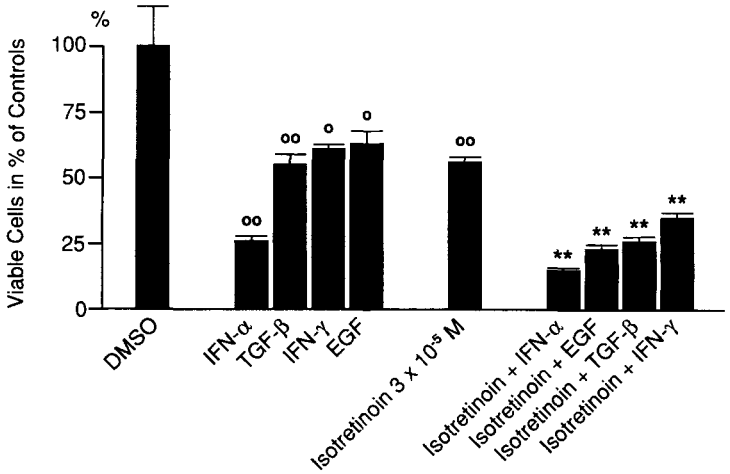

Fig. 4. Inhibition of proliferation in SCC4 cells by isotretinoin (13-cis RA) or cytokines and their combination. Cytokine concentrations: IFNα, 1000 U/ml; TGF-β, 3 ng/ml; IFNγ, 100 U/ml; EGF, 20 ng/ml. $^{o}P < 0.05$, $^{oo}P < 0.005$ by comparison with the DMSO control (Student's *t*-test). $^{**}P < 0.005$ by comparison with isotretinoin and cytokines given alone (Welch-Aspin-test). Reproduced from Bollag et al. [1992] with permission of the publisher, Elsevier Scientific Publications Ireland, Ltd.

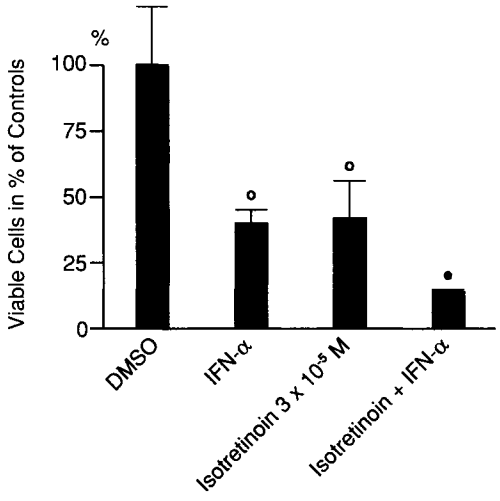

Fig. 5. Inhibition of proliferation in SCC 15 cells by isotretinoin (13-cis RA) or IFNα and their combination. Concentration of IFNα, 1,000 U/ml. °$P < 0.05$ by comparison with the DMSO control (Student's t-test). *$P < 0.05$ by comparison with isotretinoin and IFNα given alone (Welch-Aspin-test). Reproduced from Bollag et al. [1992] with permission of the publisher, Elsevier Scientific Publications Ireland, Ltd.

VIRAL ONCOGENES

A high percentage of anogenital cancers are harboring human papillomaviruses especially HPV 16 and 18. These high risk types of HPVs are thought to be causally involved in the pathogenesis of these cancers, particularly cervical carcinoma, inducing cervical intraepithelial neoplasia which progresses to invasive carcinoma. The oncogenic potential of these HPVs has been attributed primarily to two early genes, E6 and E7. HPV 18 containing cervical carcinoma HeLa and HeLa hybrid cell lines have been used to investigate the effect of retinoids on in vitro growth and oncogene expression. In parallel with growth inhibition by all-trans RA, HPV mRNA levels were down regulated due to transcriptional repression. The decrease in E6/E7 oncogene expression was demonstrated in both tumorigenic and non tumorigenic cell lines [Bartsch et al., 1992]. Besides retinoids, interferon γ inhibited transcription of E6/E7 mRNA in human cervical epithelial cell lines immortalized by HPV 16 [Woodworth et al., 1992]. HPV 18 mRNA in HeLa cells was markedly reduced by interferons α and γ [Nawa et al., 1990]. These results demonstrate that in addition to retinoids, interferons have the capacity to inhibit HPV gene expression. It is possible that combination of retinoids and interferons will synergistically decrease HPV oncogene expression.

ANGIOGENESIS

Evidence is accumulating that angiogenesis plays an important role in growth of neoplasms as well as in their control. Tumor growth beyond a certain size, a few mm^3, is dependent on formation of new blood vessels needed to deliver oxygen and nutrients to the tumor. Compounds inhibiting angiogenesis might therefore contribute to antitumor therapy [Bicknell and Harris, 1991; Folkman, 1990, 1992; Folkman and Ingber, 1992]. Administration of anti-angiogenic drugs may lead to inhibition of tumor progression, stabilization of tumor growth, or even tumor regression. Furthermore it may prevent metastases. Support of this hypothesis has recently come from clinical investigations demonstrating that lymph node metastasis in patients with mammary carcinoma is positively correlated with increasing microvessel density in the primary breast tumor [Horak et al., 1992] and that patients with a low microvessel density in their breast tumor have a better prognosis as concerns recurrence and survival than those patients with a high microvessel density [Weidner et al., 1992]. Anti-angiogenic drugs may act by inhibition of tumor cell release of angiogenic factors, by blocking of activity of angiogenic factors or by various ways of unspecific inhibition of new capillary formation [Bicknell and Harris, 1992]. Among many other compounds retinoids [Rudnicka et al., 1991; Szmurlo et al., 1992], vitamin D analogs [Oikawa et al., 1990] as well as IFNα [Sidky and Borden, 1987] were reported to possess anti-angiogenesis properties. Further investigations have been performed recently on the effects of these compounds either given alone or in combination on tumor cell line-induced angiogenesis [Majewski et al., 1993, 1994]. A series of human papilloma virus (HPV 16 and 18) harboring cell lines, either tumorigenic or non-tumorigenic, were used. Balb/c mice were injected intraperitoneally on 5 consecutive days with the test compounds. After total body X-ray exposure the human tumor cells were injected intradermally. Three days later the newly formed blood vessels at the skin injection site were counted. All-trans RA, 13-cis RA, 9-cis RA, and acitretin decreased the angiogenic potential of the tumorigenic cell lines HeLa, SKv-e2, and SKv-l2 but did not affect angiogenesis provoked by the non-tumorigenic cell lines SKv-e1 and SKv-l1. IFNα and

$1,25(OH)_2D_3$ also inhibited tumorigenic cell line-induced angiogenesis. The combination of retinoids with IFNα [Majewski et al., 1994] (Fig. 6) as well as with $1,25(OH)_2D_3$ [Majewski et al., 1993] produced a substantially higher degree of anti-angiogenesis than these compounds given alone.

MODE OF ACTION

Development and maintenance of normal tissue depends on an adequate balance between growth and differentiation. In malignancies, this equilibrium is disturbed. There is good evidence that the antitumor activity of retinoids, cytokines and $1,25(OH)_2D_3$ is at least partially due to induction of cellular differentiation and/or inhibition of cell proliferation. However, a series of other properties of these classes of compounds may contribute to an antitumor effect, such as inhibition of angiogenesis (see above), modulation of oncogene and suppressor gene expression, modulation of immune responsiveness, and induction of apoptosis.

Since retinoids, cytokines as well as $1,25(OH)_2D_3$ and its analogs have their own specific profile of properties, combination results in a mixture of individual, potentiating, additive, and synergistic effects. Such effects of combination would be expected to include also other antitumor therapeutics such as cytostatic agents, hormone agonists and antagonists.

Retinoids as well as $1,25(OH)_2D_3$ and its analogs are ligands to nuclear hormone receptors belonging to the same superfamily of steroid-thyroid hormone-retinoid receptors [Leid et al., 1992]. One of the retinoid receptor subclasses, the RXRs, have the particular capacity to form heterodimers with other hormone receptors including the vitamin D receptor [Yu et al., 1991; Kliewer et al., 1992; Carlberg et al., 1993]. This heterodimer formation at the level of receptors could account for productive interaction between their corresponding ligands, retinoic acids, and $1,25(OH)_2D_3$.

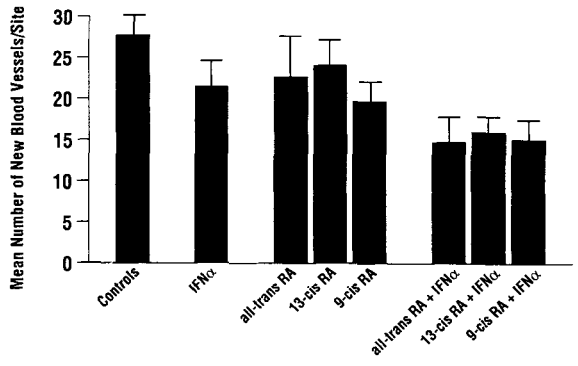

Fig. 6. Inhibition of HPV 18 harboring, tumorigenic HeLa cell-induced angiogenesis by three retinoic acid isomers (2.5 mg/kg/day i.p.), IFNα (5,000 U/kg/day i.p.), and their combination. $P < 0.001$ when RAs and IFNα were compared with controls and when combinations were compared with RAs and IFNα given alone (Student's *t*-test).

The interaction between cytokines and retinoids at the molecular level is not well documented. One potential mode of action could involve mutual modulation of the nuclear retinoid receptors and the cell membrane cytokine receptors. For example, all-trans RA and particularly 9-cis RA in the presence of IFNγ have been shown to down regulate the IFNγ receptor [Marth et al., 1993]. The subsequent internalization of IFNγ receptor complex resulting in higher intracellular IFNγ concentration could be responsible for the observed synergy between IFNγ and retinoids. The reverse situation may also be expected, i.e., treatment with cytokines might modulate the expression of retinoic acid and vitamin D receptors.

The advantage of combinations of retinoids, cytokines and $1,25(OH)_2D_3$ lies in the fact that the therapeutic activities of the different components which are based on different mechanisms of action, lead to substantially increased antitumor efficacy. In contrast, since each of these classes of compounds has a different side effect profile, combination therapy does not lead to cumulation of their individual side effects.

CONCLUSIONS

Retinoids, cytokines, 1,25-dihydroxyvitamin D_3, and analogs exert antitumor effects based on various properties of these compounds, such as induction of cell differentiation, inhibition of cell proliferation, suppression of viral oncogene expression, inhibition of angiogenesis, and others. Under experimental conditions the combination of retinoids, cytokines, $1,25(OH)_2D_3$, and analogs lead to results superior to those obtained with the single compounds. These investigations might be predictive for clinical cancer chemoprevention and chemotherapy. First positive results of clinical trials with combination therapy of retinoids and cytokines have been promising. These clinical investigations could

confirm the value of the experimental studies described in this chapter.

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