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Induction of Senescence-Associated Growth Inhibitors in the Tumor-Suppressive Function of Retinoids

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Abstract Retinoids, physiological regulators of cell growth and differentiation, are used in the treatment or chemoprevention of several malignant diseases. This class of compounds can induce growth arrest or apoptosis in tumor cells. Permanent growth arrest of retinoid-treated cells is often assumed to result from retinoid-induced differentiation. Recent studies in breast carcinoma and neuroblastoma cells demonstrated that retinoids can stop tumor cell growth through the program of senescence rather than differentiation. Retinoid-induced tumor suppression is associated with the induction of multiple intracellular and secreted growth-inhibitory proteins. Most of these proteins were also found to be upregulated in senescent cells. The induction of senescence-associated growth inhibitors appears to be an indirect effect of retinoids. Elucidation of the mechanisms responsible for the induction of growth-inhibitory genes in retinoid-treated cells should help in developing agents that would mimic the antiproliferative effect of retinoids in retinoid-insensitive cancers. J. Cell. Biochem. 88: 83–94, 2003.

Key words: retinoids; senescence; tumor suppressor genes; cancer chemotherapy

Retinoids, derivatives of vitamin A, are physiological signaling molecules that are involved in the regulation of organism development, tissue differentiation, and cell death. The effects of retinoids are mediated at the level of transcription, through binding to transcription factors formed by dimerization of retinoic acid receptors (RAR) and rexinoid receptors (RXR). These factors regulate transcription initiation by binding to retinoic acid response elements (RARE) in the promoters of retinoid-responsive genes. Retinoid receptors also affect the activity of other transcription factors through as yet unknown mechanisms. In particular, retinoid receptors are known to repress growth-stimulating transcription factor AP-1 (Jun/Fos), and AP-1 inhibition was suggested to contribute to the antiproliferative effect of retinoids [Leder et al., 1990; Chambon, 1996; Altucci and Gronemeyer, 2001].

Retinoids have been used with great success in the treatment of acute promyelocytic leukemia (APL), a disease caused by genetic rearrangements of a retinoid receptor RARα. Retinoids are also routinely used in several premalignant diseases, including leukoplakia, actinic keratosis, and cervical dysplasia, and in chemoprevention of skin cancer in patients with xeroderma pigmentosum [Altucci and Gronemeyer, 2001]. Specific retinoids have also shown encouraging results in chemoprevention trials of several other cancers, in particular breast cancer. None of the most common cancers, however, have shown so far any significant response to the therapeutic action of retinoids. The principal mechanism of retinoid resistance in human cancers is direct or indirect inactivation of RAR. In particular, the gene for retinoid receptor RARβ was shown to be a tumor suppressor, which is frequently silenced in many types of solid tumors; the loss of RARβ is responsible at least in part for the loss of retinoid sensitivity in the corresponding tumors [Li et al., 1995; Seewaldt et al., 1995; Liu et al., 1996a; Altucci and Gronemeyer, 2001].

Is it possible to exploit the physiological antiproliferative effects of retinoids in cancer

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treatment, given the ease with which tumor cells inactivate retinoid receptors. One approach involves the use of synthetic "atypical" retinoids, which appear to have both receptor-dependent and independent functions, and which also affectretinoid-resistant cells [Altucci and Gronemeyer, 2001]. It is unclear, however, if the receptor-independent activity of atypical retinoids has any relationship to the effects that are normally mediated through retinoid receptors. Another potential approach is to restore RARβ expression to tumor cells, via gene therapy or through non-specific transcription-reactivating strategies, such as inhibition of histone deacetylases or demethylation of DNA [Bovenzi and Momparler, 2001; Widschwendter et al., 2001]. The most general approach could be to bypass the retinoid receptors altogether by developing non-retinoid drugs that would mimic the effect of retinoids on signal transduction pathways responsible for the tumor-suppressive effect. The latter strategy requires an understanding of cellular events that occur downstream of the retinoid receptor action and that are responsible for the antiproliferative effect of retinoids.

In recent years, much attention has been devoted to the ability of retinoids to induce apoptosis, and candidate pathways mediating this effect have been identified [Altucci and Gronemeyer, 2001; Kucharova and Farkas, 2002]. The apoptotic activity is associated primarily with the above-mentioned "atypical" retinoids, as well as with high doses of natural retinoids. The originally described growth-inhibiting activity of natural retinoids, however, is a cytostatic effect associated with changes in cell morphology. This effect is usually attributed to retinoid-induced differentiation of tumor cells. In many cases, such as APL [Di Noto et al., 1994; Gianni et al., 1994], embryonal carcinoma [Andrews, 1984], or neuroblastoma [Linnala et al., 1997; Kohring and Zimmermann, 1998], this conclusion has been corroborated by the induction of differentiation-specific protein markers or specialized morphological structures. In other cases, however, no convincing evidence has been presented to define the retinoid response as differentiation. As described below, considerable evidence has now emerged to demonstrate that retinoids can stop the growth of tumor cells through another physiological program, cell senescence. Analysis of changes in gene expression associated with retinoid-induced growth arrest has revealed concerted induction of a group of senescence-associated genes with known growth-inhibitory or tumor-suppressive activity. The identification of genes that mediate retinoid-induced senescence should help in developing non-retinoid agents that would mimic the growth-inhibitory effect of retinoids.

TUMOR CELL SENESCENCE AS A DETERMINANT OF TREATMENT RESPONSE

Cell senescence is a physiological process that leads to irreversible growth arrest, accompanied by characteristic phenotypic changes (such as enlarged and flattened cell shape, increased granularity, and induction of senescence-associated β-galactosidase activity, SA-β-gal). Senescence was originally described in normal human cells explanted in culture; such cells undergo only a limited number of cell divisions prior to permanent growth arrest [Hayflick and Moorhead, 1961]. This gradual process of "replicative senescence" is now known to result from shortening of telomeres at the ends of the chromosomes [Campisi, 2000]. More recently, senescence was also shown to occur as a rapid process that does not involve telomere shortening. This "accelerated senescence" is triggered by such factors as DNA damage or expression of mutant Ras [Di Leonardo et al., 1994; Serrano et al., 1997; Robles and Adami, 1998]. Growth arrest in both replicative and accelerated senescence of normal cells is mediated by the activation of p53, which then induces a cyclin-dependent kinase (CDK) inhibitor $p21^{Waf1/Cip1/Sdi1}$ thus producing cell cycle arrest. The levels of p21 decrease after the establishment of growth arrest, but another CDK inhibitor, $p16^{Ink4A}$ becomes constitutively upregulated. Continuous p16 expression is believed to be responsible for the maintenance of growth arrest in normal senescent cells [Alcorta et al., 1996; Stein et al., 1999].

Cell senescence, like apoptosis, is believed to be a natural anti-carcinogenic program [Campisi, 2000]. Indeed, the process of carcinogenesis involves events that inhibit senescence. These include activation of telomerase, an enzyme that extends telomeres and thereby prevents replicative senescence, and inactivation of tumor suppressors p53 and p16, which mediate both replicative and accelerated senescence. Nevertheless, tumor cells, which as a

rule have short telomeres and carry senescence-promoting mutations (such as mutant RAS), can be induced to undergo accelerated senescence. This can be achieved by ectopic overexpression of tumor suppressor genes (such as p53, RB, p16, or p21), or by inhibition of telomerase [Shammas et al., 1999] or other senescence-suppressing oncogenes. For example, inhibition of papillomavirus oncoproteins E6 and E7 in cervical carcinoma induced rapid senescence in almost 100% of the cells [Goodwin et al., 2000]. Our laboratory has found that treatment of tumor cells with various chemotherapeutic drugs or ionizing radiation induces the senescent phenotype in many of the treated cells. Such cells remain intact but they do not divide or form colonies [Chang et al., 1999a]. Chemotherapy-induced senescence was also demonstrated in xenograft models [Roninson et al., 2001] and in clinical samples of breast cancer [te Poele et al., 2002]. Tumor senescence, along with apoptosis, was shown in a recent study to determine in vivo response to chemotherapy in a transgenic mouse model of B-cell lymphoma [Schmitt et al., 2002].

In the study of Schmitt et al. [2002], treatment-induced senescence of mouse lymphoma cells was undetectable in the absence of either p53 or p16. In human tumor cell lines, however, drug-induced senescence readily develops in the absence of p16, and it is diminished but not abolished by the loss of p53 or p21 [Chang et al., 1999b]. This suggested that some genes other than p53, p21, or p16 are likely to play a role in accelerated senescence of tumor cells. Indeed, cDNA microarray analysis showed that doxorubicin-induced senescence of human colon carcinoma cells is associated with sustained induction of multiple growth-inhibitory genes, including several tumor suppressors. These include intracellular growth inhibitors, such as BTG1, BTG2, and EPLIN, as well as secreted proteins with growth-suppressing activity, such as Maspin, MIC-1, or IGFBP-6 [Chang et al., 2002].

On the other hand, some of the genes upregulated in doxorubicin-induced senescence encode secreted factors with anti-apoptotic, mitogenic, and angiogenic functions [Chang et al., 2002]. Expression of these genes is likely to account for paracrine tumor-promoting activities that were associated with different forms of senescence in human fibroblasts [Krtolica et al., 2001]. The induction of tumor-promoting factors is mediated in part through p21, and p21 expression alone is sufficient to induce such genes and their associated paracrine activities [Chang et al., 2000]. p21 induction is a common response to DNA-damaging agents and some other chemotherapeutic drugs, and the side effects of p21 induction need to be taken into account when considering the effects of tumor senescence on the outcome of conventional chemotherapy.

RETINOID-INDUCED SENESCENCE IN HUMAN TUMOR CELL LINES

Given the ease with which drug-treated tumor cells undergo senescence, it was natural to consider if retinoids could have the same effect. This possibility was first investigated in MCF-7 breast carcinoma cells treated with all-*trans*-retinoic acid (RA) [Chang et al., 1999a]. To minimize the cytotoxic effect of retinoids, MCF-7 cells were treated with a low (100 nM) dose of RA. RA-treated cells started growing slower than untreated cells between days 4 and 6, and showed a small (20%) decrease in cell number between days 6 and 9 (Fig. 1A). In this latter period, no cell division could be detected by a flow cytometric assay, indicating that the effect of RA was primarily cytostatic [Chang et al., 1999a]. This growth arrest was largely irreversible, since 7-day exposure to 100 nM RA decreased colony formation in drug-free media by 90% [Dokmanovic et al., 2002]. RA-induced growth arrest was accompanied by senescence-like changes in cell morphology (enlarged and flattened cells, increased granularity) and by a drastic increase in SA-β-gal expression (Fig. 1B), which reached 84% after 8 days of treatment (Fig. 1A). The combination of morphological changes, SA-β-gal induction, and irreversible growth arrest indicated that RA-treated MCF-7 cells were undergoing senescence. The same study [Chang et al., 1999a] showed that induction of the senescent phenotype by retinoids is not limited to cell culture. Thus, SA-β-gal expression was also induced by in vivo treatment of mice carrying a xenograft of MCF10AneoT transformed mammary epithelial cells with an atypical retinoid fenretinide [4-(Hydroxyphenyl) retinamide, 4-HPR].

In a more recent study, Wainwright et al. [2001] compared RA-induced senescence and differentiation in human neuroblastoma cells. Remarkably, two otherwise indistinguishable

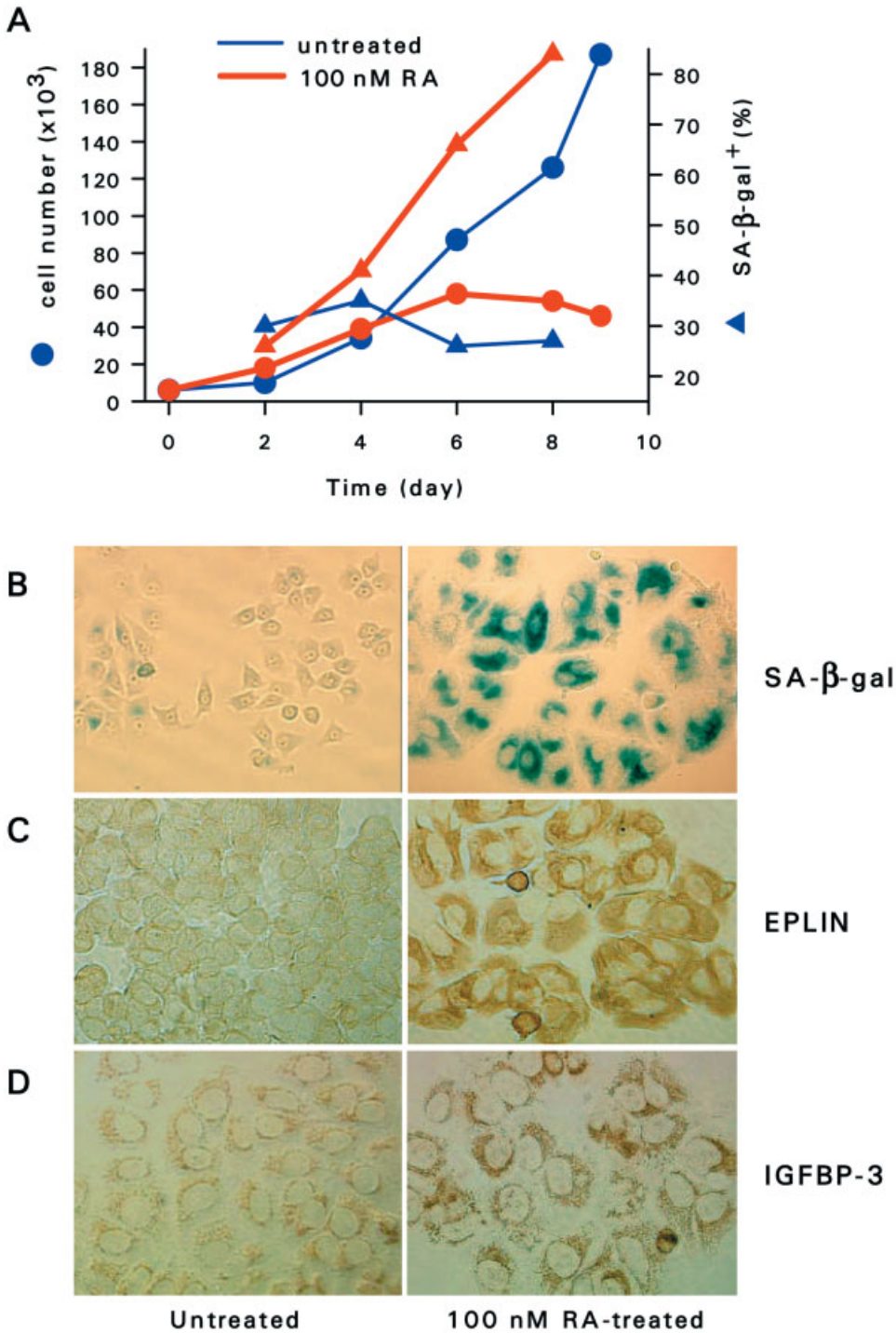

Fig. 1. Retinoic acid-induced senescence in MCF-7 breast carcinoma cells. **A**: Time course of changes in cell number (circles) and percentages of SA-β-gal+ cells (triangles) for MCF-7 cells, untreated (thin blue lines) or treated with 100 nM RA (thick red lines) (from Chang et al., 1999a). **B**: SA-β-gal staining of untreated MCF-7 cells and cells exposed to 100 nM RA for 8 days (from Chang et al., 1999a). **C**: Immunostaining for EPLIN in untreated MCF-7 cells and in cells treated with 100 nM RA for 5 days (from Dokmanovic et al., 2002). **D**: Immunostaining for IGFBP-3 in untreated MCF-7 cells and in cells treated with 100 nM RA for 5 days (from Dokmanovic et al., 2002).

subclones of the neuroblastoma cell line SK-N-SH showed different morphological responses to RA. In a subclone designated SH-N, RA treatment induced neuronal differentiation, characterized by extensive neurite outgrowths, and induction of differentiation markers (neurofilaments 68 and 160). In another subclone, SH-F, RA treatment induced characteristic features of senescence, as it transformed the small neuroblastic cells into large, flattened, epithelium-like cells, which were characterized by the accumulation of SA-β gal marker by day 7 of treatment. Although both cell lines were growth arrested by RA, only the senescing SH-F cells downregulated Cyclin D1 expression, and their growth arrest developed more rapidly than in the differentiating SH-N cells.

Wainwright et al. [2001] also compared the expression of several CDK inhibitors in SH-N and SH-F cell lines and found two major differences. One of them was that $p16^{Ink4A}$ and a related CDK inhibitor $p18^{Ink4B}$ were expressed only in SH-F but not in SH-N cells, and expression of these proteins in SH-F was mildly increased by RA (p16 upregulation, however, was only transient). The second difference was that RA treatment increased the levels of p21 in the differentiating SH-N cells, but it *decreased* p21 expression in SH-F cells undergoing senescence. Transfection of SH-N with either p21 or p16 inhibited cell growth, but only p21 induced differentiation in this subclone [Wainwright et al., 2001]. These results suggested that p21, which plays multiple roles in different cellular processes [Dotto, 2000], may be a key switch between retinoid-induced senescence and differentiation. Interestingly, RA treatment of MCF-7 cells also decreases p21 expression [Zhu et al., 1997]. p21 downregulation in both of the characterized systems of retinoid-induced senescence stands in sharp contrast to the senescence induced by conventional chemotherapeutic drugs, where p21 expression is usually increased.

RETINOID-INDUCED SENESCENCE IS ASSOCIATED WITH THE INDUCTION OF MULTIPLE GROWTH-INHIBITORY GENES

Genes that are directly induced by retinoids typically increase their expression within 12 h of retinoid treatment, but the onset of growth arrest and senescence in RA-treated MCF-7 cells requires at least 4 days (Fig. 1A). This growth response seems likely therefore to be mediated by indirect transcriptional effects of retinoids. To identify genes that maintain senescence in retinoid-treated tumor cells, we have used cDNA microarray hybridization to compare gene expression between untreated MCF-7 cells and cells treated with retinoids for 5 days, the period of time required for pronounced growth inhibition and expression of the senescent phenotype [Dokmanovic et al., 2002]. cDNA microarray hybridization was followed by reverse transcription-PCR assays for 47 genes that showed the biggest changes in the microarray. This analysis revealed that 13 genes showed a major (5–10 fold or higher) increase in their RNA levels after 5 days of RA treatment, whereas changes in the expression of other genes (either upregulated or downregulated) were only minor. All 13 of the strongly affected genes were induced both by RA and by atypical retinoid fenretinide.

A very high fraction (4/13) of the strongly induced genes encode growth-inhibitory proteins, some of which have been implicated in other models of cell senescence. The nature of these genes (described in the next section) suggests that they are directly involved in retinoid-induced growth arrest. Some of the other genes induced in MCF-7 cells are involved in RA synthesis, the proteasome-mediated protein degradation, and cell adhesion. Interestingly, while some of the induced genes encode markers of hematopoietic differentiation (a well-known effect of retinoids), none of them have been associated with epithelial differentiation, providing additional evidence that retinoid-induced arrest of MCF-7 carcinoma cells represents senescence rather than differentiation [Dokmanovic et al., 2002].

A number of other studies have demonstrated that retinoid treatment of tumor cells upregulates the expression of tumor-suppressing proteins. Table I lists 18 growth-inhibitory genes that have been shown in the literature to be induced in solid tumor or leukemia cells by retinoid treatment, including four genes identified in our study. Interestingly, five genes in Table I encode secreted growth-inhibitory proteins, which inhibit the growth not only of the expressing cells but also their neighbors. Remarkably, 14 of 18 genes in Table I, including all five secreted inhibitors, have been associated with cell senescence, as described in the next section. It remains to be determined if the

TABLE I. Retinoid-Inducible Growth-Inhibitory Genes

Gene	Inducing retinoids	Tumor type	References
Senescence-associated growth inhibitors (intracellular)			
RARβ	RA, 9-*cis* RA, 13-*cis* RA, 4-HPR	Squamous cell carcinoma, breast carcinoma, neuroblastoma, hepatoma, lung carcinoma, cervical carcinoma, teratocarcinoma, melanoma, colon carcinoma, pancreatic carcinoma, esophageal carcinoma, glioma	de The et al. [1989]; Nervi et al. [1991]; Bartsch et al. [1992]; Clagett-Dame et al. [1993]; Kurie et al. [1993]; Swisshelm et al. [1994]; Lotan [1994]; Xu et al. [1994]; Redfern et al. [1990]; Spanjaard et al. [1995]; Kazmi et al. [1996]; Agarwal et al. [1996]; Zugmaier et al. [1996]; Xu et al. [1999]; Carpentier et al. [1999]; Lee et al. [2000]
EPLIN β	RA, 4-HPR	Breast carcinoma	Dokmanovic et al. [2002]
FAT-10	RA, 4-HPR	Breast carcinoma	Dokmanovic et al. [2002]
BTG-1	RA	APL	Liu et al. [2000a]
p73	RA	Neuroblastoma	De Laurenzi et al. [2000]
$p27^{Kip1}$	RA, 9-*cis* RA	Neuroblastoma, astrocytoma, lung carcinoma, ovarian carcinoma, oral squamous cell carcinoma, myeloblastic leukemia	Weber et al. [1999]; Dirks et al. [1997]; Matsuo and Thiele [1998]; Hsu et al. [2000]; Dimberg et al. [2002]; Pomponi et al. [1996]; Hayashi et al. [2000]
$p18^{Ink4C}$	RA	Myeloblastic leukemia, neuroblastoma	Shimizu and Takeda [2000]; Wainwright et al. [2001]
$p16^{Ink4A}$	RA	Neuroblastoma, ovarian carcinoma	Wainwright et al. [2001]; Zhang et al. [2001]
$p21^{Waf1/Cip1/Sdi1}$	RA, CD 437, 9-*cis* RA	Myeloma, APL, melanoma, oral squamous cell carcinoma, gastric carcinoma, APL, lung carcinoma, hepatoma	Chen et al. [1999]; Naka et al. [1997]; Liu et al. [1996b]; Adachi et al. [1998]; Li et al. [1998]; Casini and Pelicci [1999]; Sun et al. [1999]; Hsu et al. [1999]; Hayashi et al. [2000]; Demary et al. [2001]
Senescence-associated growth inhibitors (secreted)			
IGFBP-3	RA, 4-HPR, TTNPB	Breast carcinoma, squamous cell carcinoma, prostatic adenocarcinoma, hepatocellular carcinoma, cervical carcinoma	Goossens et al. [1999]; Murakami et al. [2000]; Gucev et al. [1996]; Hwa et al. [1997]; Adamo et al. [1992]; Andreatta-Van Leyen et al. [1994]; Le et al. [2002]
IGFBP-6	RA, 9-*cis* RA, 13-*cis* RA	Colon carcinoma, embryonal carcinoma, osteosarcoma, neuroblastoma, breast carcinoma, SV-40 transformed fibroblasts	Freemantle et al. [2002]; Kim et al. [2002]; Sheikh et al. [1993]; Yan et al. [2001]; Martin et al. [1994]; Zhou et al. [1996]; Chambery et al. [1998]; Babajko and Binoux [1996]
IGFBP-7/mac25	RA	Mammary carcinoma	Swisshelm et al. [1995]
βig-h3	RA, 4-HPR	Breast carcinoma	Dokmanovic et al. [2002]
TGFβ-1	RA	U937 leukemia	Defacque et al. [1999]
Other growth inhibitors (intracellular)			
TIG-3/RIG-1	RA	Breast carcinoma, gastric carcinoma	Huang et al. [2000]; DiSepio et al. [1998]
Drg-1	LG268	Colon carcinoma	Guan et al. [2000]
Nm23-H1	RA	Hepatocellular carcinoma	Liu et al. [2000b]
ASB-2	RA	APL	Guibal et al. [2002]

remaining four inhibitors (putative tumor suppressors TIG-3/RIG-1 and DRG-1, metastasis suppressor nm23-H1, and SOCS family protein ASB-2) are also overexpressed in any forms of senescence.

We have screened the promoter sequences of all the growth-inhibitory human genes in Table I (except for DRG-1, the promoter of which is currently absent from the human genome database) for the presence of retinoid response elements (RARE). We have found a well-defined RARE sequence in only one gene, RARβ (transcription of which is known to be induced by retinoids via another receptor, RARα). In addition, the promoter of IGFBP-7 gene contains two potential RARE half sites (AGGTCA) about 1,450 bp upstream of the transcription start site, but this remote position and an unusual inverse orientation of the two half sites make it unlikely that these are functional RARE sequences. Similarly, we have previously reported that the promoter of only one of 13 genes that were strongly induced by retinoids in MCF-7 cells contained RARE, and this gene was induced more rapidly than the other 12 genes (including all four growth inhibitors identified in this study) [Dokmanovic et al., 2002]. These observations suggest that induction of the

majority of growth-inhibitory genes is an indirect effect of retinoids.

NATURE OF SENESCENCE-ASSOCIATED RETINOID-INDUCIBLE GROWTH INHIBITORS

The first gene in Table I is retinoid receptor RARβ, a tumor suppressor and a key determinant of tumor cell response to retinoids. In particular, RARβ2 isoform is upregulated in senescent dermal fibroblasts [Lee et al., 1995] and mammary epithelial cells [Swisshelm et al., 1994], suggesting a role for RARβ in replicative senescence. Expression of RARβ not only sensitizes cells to retinoids but also has its own growth-inhibitory effect [Si et al., 1996].

One of the intracellular proteins induced by retinoids in MCF-7 cells is Epithelial Protein Lost in Neoplasm (EPLIN), an actin-binding LIM domain protein, which is expressed in primary epithelial cells but downregulated in different types of carcinomas [Maul and Chang, 1999]. Re-expression of EPLIN is associated with the induction of senescence not only in retinoid-treated MCF-7 cells (as illustrated by immunohistochemical staining in Fig. 1C) but also in doxorubicin-treated HCT116 colon carcinoma cell line [Chang et al., 2002]. Another senescence-associated growth inhibitor identified in the latter study is the tumor suppressor BTG1, which was found by Liu et al. [2000a] and Zhang et al. [2001] to be induced by RA in APL cells. Still another retinoid-inducible tumor suppressor is p73, a p53-related gene, overexpression of which induces senescence in bladder carcinoma cells [Fang et al., 1999]. One of the genes upregulated in retinoid-induced senescence of MCF-7 cells encodes an ubiquitin-like protein FAT10. FAT10 interacts with one of the components of mitotic spindle checkpoint [Liu et al., 1999], and we have shown that FAT10 inhibits MCF-7 cell growth [Dokmanovic et al., 2002].

The remaining senescence-associated intracellular growth inhibitors in Table I are CDK inhibitors p21, p16, p18, and p27. p16 and p21 play a key role in replicative and accelerated senescence of normal cells, and p27 appears to mediate some of the pathways of accelerated senescence [Bringold and Serrano, 2000]. As mentioned above, p16 and p18 are upregulated in retinoid-induced senescence of SH-F neuroblastoma cells [Wainwright et al., 2001]. In contrast, p21, as mentioned above, is downregulated in SH-F and MCF-7 cells that undergo RA-induced senescence, and p21 induction in SH-N neuroblastoma is associated with differentiation rather than senescence.

Three of the five secreted growth inhibitors in Table I belong to the insulin-like growth factor (IGF)-binding protein (IGFBP) family of proteins, which modulate the binding of IGFs to their receptors. The best known of these is IGFBP-3, which was shown to be induced by different retinoids in many types of tumor cells. Figure 1D illustrates the induction of IGFBP-3 in RA-treated MCF-7 cells. Overexpression of the IGFBP-3 gene or addition of the IGFBP-3 protein to culture media inhibit the growth of tumor cells. This inhibition is associated with both cell cycle arrest and apoptosis, which is at least in part IGF-independent [Hong et al., 2002]. IGFBP-3 is strongly overexpressed in senescent human fibroblasts [Goldstein et al., 1991] and prostate epithelial cells [Schwarze et al., 2002]. Another retinoid-inducible protein of the same family, IGFBP-6, is upregulated in doxorubicin-induced senescence of colon carcinoma cells [Chang et al., 2002]. Of special interest is another retinoid-inducible member of this family, IGFBP-7, also known as mac25 or IGFBP-rP1. Expression of this protein in MCF-7 cells was recently shown to induce not only growth arrest but also the senescent phenotype [Wilson et al., 2002]. Another retinoid-inducible secreted protein, TGFβ-1, is induced in many types of senescent cells, and its induction was shown to mediate the development of the senescent phenotype in human fibroblasts treated with hydrogen peroxide [Frippiat et al., 2001]. The last protein in this group is an extracellular matrix protein βig-h3, which is upregulated in RA-induced senescence of MCF-7 cells. βig-h3 is a TGFβ-inducible gene which is expressed in normal but not in transformed human fibroblasts [Schenker and Trueb, 1998], and its expression inhibits the tumorigenicity of Chinese hamster ovary cells [Skonier et al., 1994].

SUMMARY AND FUTURE DIRECTIONS

It has now become apparent that activation of the program of cell senescence is one of the mechanisms of tumor suppression by retinoids. Future studies will undoubtedly provide many other examples of retinoid-induced senescence and will allow us to compare the relative

contributions of senescence and differentiation to the antiproliferative effect of retinoids. Retinoid-induced senescence of tumor cells shares many similarities with senescence induced by DNA-damaging chemotherapeutic drugs or radiation, both at the phenotypic level and at the level of specific growth-inhibitory genes that are upregulated in both types of senescence. On the other hand, there are important differences between the characterized systems of retinoid-induced and damage-induced senescence of tumor cells. Both replicative senescence and damage-induced accelerated senescence are associated with the induction of CDK inhibitor p21, which in its turn upregulates a set of genes that encode secreted factors with mitogenic, anti-apoptotic and angiogenic activities. p21 induction is likely to be responsible at least in part for the paracrine tumor-promoting functions associated with senescent cells. Drug- or radiation-induced senescence, however, can also occur in the absence of p21, albeit at a diminished rate [Chang et al., 1999b]. In contrast to damage-induced senescence, p21 is downregulated by RA treatment in both NH-F neuroblastoma and MCF-7 breast carcinoma cell lines. Remarkably, none of the genes that we have found to be induced in RA-treated MCF-7 cells encode proteins with known tumor-promoting functions, whereas some of the proteins induced in these cells have paracrine tumor-suppressing effects. This suggests that retinoid-induced senescence represents an especially desirable form of tumor suppression.

The identification of growth-inhibitory genes that are upregulated in retinoid-induced senescence opens potential venues towards developing non-retinoid drugs that will induce the same type of senescence in tumor cells. Two lines of evidence suggest that it should be possible to find such agents. The first argument is that the retinoid-inducible growth inhibitors, with the exception of RARβ, have no apparent RARE sites in their promoters. These genes appear to be induced by retinoids through an indirect mechanism, which is likely to be susceptible to other types of inducers. One well known indirect effect of retinoids is downregulation of proliferation-associated transcription factor complex AP-1 [Altucci and Gronemeyer, 2001], but at present we have no evidence to relate the induction of retinoid-inducible growth inhibitors to the AP-1 function. The second argument is that most of retinoid-inducible growth inhibitors are upregulated in senescent cells that have never been exposed to retinoids, and some of these inhibitors (e.g., EPLIN, BTG1, IGFBP-6) are inducible by conventional chemotherapeutic drugs. Elucidation of the regulatory pathways responsible for the induction of senescence-associated growth inhibitors in retinoid-treated cells and development of high-throughput screening systems for the induction of such inhibitors will enable us to explore this novel strategy for stopping the growth of tumor cells.

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