Perspectives in Cancer Research

Role of Retinoids in Differentiation and Carcinogenesis

Michael B. Sporn and Anita B. Roberts

Laboratory of Chemoprevention, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Maryland 20205

It has been known for more than 50 years that retinoids, the family of molecules comprising both the natural and synthetic analogues of retinol, are potent agents for control of both cellular differentiation and cellular proliferation (70). In their original classic paper describing the cellular effects of vitamin A deficiency in the rat, Wolbach and Howe clearly noted that there were distinct effects on both differentiation and proliferation of epithelial cells. During vitamin A deficiency, it was found that proper differentiation of stem cells into mature epithelial cells failed to occur and that abnormal cellular differentiation, characterized in particular by excessive accumulation of keratin, was a frequent event. Furthermore, it was noted that there was excessive cellular proliferation in many of the deficient epithelia. Although the conclusion that an adequate level of retinoid was necessary for control of normal cellular differentiation and proliferation was clearly stated in the original paper by Wolbach and Howe, a satisfactory explanation of the molecular mechanisms underlying these effects on both differentiation and proliferation still eludes us more than 50 years later.

It was inevitable that the basic role of retinoids in control of cell differentiation and proliferation would eventually find practical application in the cancer field, and there have been great advances in this area, particularly for prevention of cancer. Many studies have shown that retinoids can suppress the process of carcinogenesis in vivo in experimental animals (for reviews, see Refs. 7, 33, 51, 54, 56, and 57), and these results are now the basis of current attempts to use retinoids for cancer prevention in humans. Furthermore, there is now an extensive literature on the ability of retinoids to suppress the development of the malignant phenotype in vitro (for reviews, see Refs. 6, 8, 30, and 31), and these studies corroborate the use of retinoids for cancer prevention. Finally, most recently, it has been shown that retinoids can exert effects on certain fully transformed, invasive, neoplastic cells, leading in certain instances to a suppression of proliferation (30) and in other instances to terminal differentiation of these cells, resulting in a more benign, nonneoplastic phenotype (10, 11, 60, 62). Even though there are many types of tumor cells for which this is not the case (33, 52) (indeed, at present there are only a limited number of instances in which such profound effects of retinoids on differentiation and proliferation of invasive tumor cells have been shown), this finding nevertheless has highly significant implications for the problem of cancer treatment. It emphasizes that in many respects cancer is fundamentally a disease of abnormal cell differentiation (36, 44), and it raises the possibility that even invasive disease may eventually be controlled by agents which control cell differentiation rather than kill cells. Since carcinogenesis is essentially a disorder of cell differentiation, the overall scientific problem of the role of retinoids in either differentiation or carcinogenesis is essentially the same problem and will be considered as a single problem in this brief review.

Major Problems Relating to Retinoids and Cancer

In the broadest sense, there are 2 major domains relating to retinoids and the cancer problem: (a) the practical development and use of retinoids for either cancer prevention or treatment; and (b) the elucidation of the cellular and molecular mechanisms underlying the first domain. The first domain has attracted a great deal of attention in the past 5 years and requires the coordinated efforts of synthetic organic chemists, cell biologists, investigators in experimental carcinogenesis and chemotherapy, pharmacologists, toxicologists, and clinical investigators in order to synthesize new retinoids, test them both in vitro and in vivo for useful biological activity, establish their pharmacokinetic and toxicological properties, and then bring the best new retinoids to clinical trial for either prevention or treatment of specific types of cancer. This is a problem of immense scope, complexity, and expense, which is currently being pursued with vigorous interest throughout the world. We have reviewed some of the key issues in this first domain in previous articles (54, 56, 57) and will not discuss them further at this point. Instead, we will focus the rest of this short review on the problem of cellular and molecular mechanisms. Studies in this area are not only of great theoretical interest but should also facilitate the practical development and use of retinoids for prevention and treatment of cancer. Elucidation of the mechanism of action of retinoids may also lead to new applications for their use. It is reasonable to suggest that retinoids may find applications in the prevention or treatment of diseases other than cancer, the pathogenesis of which involves abnormalities of cell differentiation and/or proliferation (55). In terms of the scientific challenge, it is again worth emphasizing that the problem of cellular and molecular mechanism is still unsolved more than 50 years after the initial description of the overall biological activity of the retinoids. The problem of the mechanism of action of retinoids may be studied at 3 levels, namely, in the whole animal, at the cellular level, and finally at the molecular level, which we shall now consider in turn, using effects on both differentiation and carcinogenesis as markers.

Mechanism of Action of Retinoids in Differentiation and Carcinogenesis Studied in the Whole Animal

Although the earliest studies on retinoid deficiency in the whole animal emphasized its effects on epithelial cell differentiation and proliferation, the possibility that retinoid deficiency caused abnormalities in nonepithelial cells derived from mesenchymal elements was also noted by several careful investigators. Indeed, in the 1920s, it was reported that there was a reduction in hematopoietic cells in the bone marrow of vitamin A-deficient...
animals (21, 70). In the 1930s and 1940s, many studies were performed on the need for retinoids for proper bone formation (34, 39), and there was detailed investigation of the control of osteoblasts and osteoclasts (cells derived from mesenchyme) by retinoids (34). However, in the ensuing years, there was a much greater emphasis on studies on the role of retinoids in control of epithelial cell differentiation and proliferation, and the dogma that retinoids were selectively involved in the control of epithelial cells and were of relatively minor importance with respect to cells of mesenchymal origin became scientific folklore.

In contrast, in the area of experimental embryology and teratology, there accumulated an impressive body of information which indicated that retinoids had significant, selective teratogenic action on cells of mesenchymal origin in rat, mouse, hamster, and chick embryos; we have reviewed these data elsewhere (58). Particularly striking were the effects of either retinol deficiency or retinoic acid excess on the development of the very early vascular system of either the chick or the rat embryo. In the 1-day-old chick embryo, retinol deficiency causes failure of mesenchymal cells to proliferate and differentiate to form the early vascular system (Refs. 66, 67; Fig. 1); treatment of rat or chick embryos with excess retinol or retinoic acid has similar effects (40). Furthermore, in the retinoid-deficient chick embryo, normal development of the vascular system can be restored by injection of appropriate amounts of various retinoids, including esters of retinoic acid (66, 67). These results indicate a very stringent requirement for retinoids, with either deficiency or excess leading to abnormal development of tissues derived from primitive mesenchyme. The effects of retinoids on the developing vascular system of the early embryo appear to be quite selective, since many other cell types do not appear to be affected to anywhere near the same extent (58, 66, 67). In the early embryo, a common stem cell type has long been believed to be a precursor to both blood cells themselves and those cells which will form the walls of the earliest blood vessels (50). The preceding observations, made with only the simplest of morphological techniques, suggest that retinoids play a role in controlling the proliferation and differentiation of those mesenchymal precursor cells or their early progeny; more recent work, using sophisticated cell culture and recombinant DNA techniques, has added important further information, as will be discussed later.

Mechanistic studies in the whole animal on the role of retinoids in prevention of carcinogenesis have dealt largely with the prevention of epithelial carcinogenesis. There are especially convincing data on the efficacy of many different retinoids in prevention of skin, breast, and bladder cancer in experimental animals (5, 7, 33, 37, 38, 57, 59, 65). Overall, these studies suggest that retinoids exert a hormone-like control of either cell proliferation or cell differentiation. However, in the whole animal, it is extremely difficult to separate these 2 parameters; they are intimately linked with each other. Some investigators have stressed the role of retinoids as antiproliferative agents (38), while others have emphasized that the role of retinoids in control of differentiation, rather than proliferation, may be more important (4). Whole-animal studies do not easily lend themselves to separate analysis of these 2 parameters. Indeed, the problem of the separation of the effects of retinoids on cell proliferation, as contrasted to effects on cell differentiation, may turn out to be more semantic than real, once a complete genetic analysis, using recombinant DNA methods, is available. The key problem is not to deal with the semantics of whether retinoids preferentially affect cell proliferation or cell differentiation but to identify the specific genes, the function of which is ultimately controlled by retinoids, either directly or indirectly. This problem cannot be solved in the whole animal; it requires isolated cellular systems and modern methods of molecular analysis. We will now discuss the application of studies in these areas to understanding the role of retinoids in differentiation and carcinogenesis.

### Cellular Mechanism of Action of Retinoids in Differentiation and Carcinogenesis

Significant advances in understanding the mechanism of action of retinoids did not occur until in vitro systems were used as experimental tools. The development of methods for organ culture of tissues, such as the skin and the prostate, in which retinoids are particularly active, was a major advance. In the 1950s, the classic studies of Fell and Mellanby (20) showed that the differentiated phenotype of chick epidermis in organ culture could be changed from keratinized to mucus producing by treatment with retinol or retinyl acetate. In cultures treated with retinoids, the keratinizing cells of the epidermis disappeared and were replaced by mucus-producing cells, and in some instances even by ciliated cells, which are not found in normal skin (20). These organ culture experiments were essentially the reverse of those performed by Wolbach and Howe in the 1920s in the whole animal. In the Wolbach-Howe studies, retinoid deficiency caused disappearance of normal mucociliary epithelium, with replacement by keratinizing cells (keratinizing squamous metaplasia); in the Fell-Mellanby experiments, retinoid excess caused disappearance of normal keratinizing epithelium, with replacement by mucus and ciliated cells (mucus metaplasia).

The next significant advance in this area also used organ culture methodology. Lasnitzki (26), working in the same laboratory as Fell and Mellanby, was able to show that the premalignant phenotype of mouse prostate glands that had been treated with the carcinogen, 3-methylcholanthrene, could be altered by retinoids. The atypical epithelial cells that were induced by the carcinogen disappeared upon retinoid treatment of the organ cultures, and they were replaced by cells with more normal morphology (26). The effects of the retinoids were to suppress abnormal cellular differentiation that had been induced by the carcinogen in the epithelium of the prostate gland and to restore a more normal pattern of epithelial differentiation. In other organ culture studies, Lasnitzki (27) also made the important observation that there were significant morphological similarities between vitamin A-deficient prostatic epithelium and prostatic epithelium in cultures that had been treated with methylcholanthrene; these studies provide further evidence for the concept that the mechanisms of action of retinoids in both differentiation and carcinogenesis are closely linked.

In spite of the advances that were made in the above experiments, organ culture methods have definite liabilities for analysis of mechanism; they use mixed-cell populations, from which it is very difficult, if not impossible, to obtain replicate samples of homogeneous cells. The introduction of cell culture methodology to studies of retinoid mechanism was therefore of great importance and now is allowing molecular investigation of the role of retinoids in differentiation and carcinogenesis. In contrast to the organ culture studies, in which the emphasis was on the role of...
retinoids in control of epithelial differentiation, cell culture studies have emphasized the role of retinoids in cells of mesenchymal origin, if only because such cells are grown more easily in culture. One may suppose that, as better systems are developed for epithelial cell culture, there will be increasing investigation of retinoids in many different types of epithelium using these methods; this has already happened with epidermal cell culture (72).

Continuous cell lines of mesenchymal origin have been widely used to study the effects of retinoids on both differentiation and carcinogenesis; the cell lines which have been used are both neoplastic and nonneoplastic. The experiments which opened up this area of investigation were those of Merriman and Bertram (35) and Harisiadis et al. (24), which showed that retinoids can act directly on nonneoplastic cells to suppress the process of malignant transformation induced by either chemicals or radiation. In the case of the experiments done with suppression of chemical carcinogenesis, it was clearly shown that retinoids were effective in suppressing transformation even when they were applied to cells a full week after original exposure of the cells to carcinogen. Whatever the genetic damage caused by the carcinogen, it had already occurred. The role of the retinoids in these experiments was thus clearly shown to be a suppressor of the expression of the malignant phenotype in cells that had been previously initiated by a carcinogen (35). Furthermore, in these experiments, continuous presence of the retinoids was required to suppress the malignant phenotype; removal of the retinoids from the culture allowed expression of the transformed state.

Retinoids can also change the differentiation of invasive neoplastic cells growing in either monolayer or suspension culture. The most striking example of this phenomenon is the induction of terminal differentiation in murine F9 teratocarcinoma cells (60, 62) or human promyelocytic leukemia cells (Refs. 10 and 11; Fig. 2); in these cases, the differentiated phenotype is drastically changed from neoplastic to nonneoplastic, and proliferation of the induced cells is permanently suppressed. In the F9 system, retinoids induce terminal differentiation of teratocarcinoma stem cells to cells which resemble parietal endoderm; a variety of new proteins is induced in the differentiated cells (62, 63). In the human promyelocytic leukemia system, retinoids can induce terminal differentiation of malignant leukemia cells, leading to formation of morphologically mature granulocytes, which have functional markers of the mature neutrophil (10, 11); these results have been obtained with the established HL60 cell line (11), as well as with primary cultures of other promyelocytic leukemia cells (10). These studies with neoplastic leukemia cells in turn have had a major influence on studies on possible effects of retinoids on normal myeloid differentiation. Since some of the leukemias may be viewed as diseases in which there is a block or arrest in normal myeloid differentiation and maturation (14, 23) and since retinoids can apparently overcome this block in certain leukemia cells, it has been suggested that retinoids may also be involved in normal hematopoiesis (11, 18).

The mechanism of all of these effects of retinoids, whether they be to alter the differentiated phenotype in nonneoplastic or preneoplastic epithelial cells in organ culture, to suppress the appearance of the neoplastic phenotype in nonneoplastic mesenchymal cells in monolayer culture, or to induce the terminally differentiated phenotype in fully neoplastic cells in monolayer culture, is not known. It is tempting to believe that there is a common mechanism (or limited number of mechanisms), which underlies all of these phenomena. In cell culture studies, as we noted before in the studies on whole animals, various investigators again have chosen to emphasize the role of retinoids in control of either cell proliferation (30) or cell differentiation (72). It would appear that retinoids control both processes and that any dispute over which is more important is relatively fruitless at present. Rather, it would seem more productive to focus on the specific molecular processes involved, to which we shall now turn.

Molecular Mechanism of Action of Retinoids in Differentiation and Carcinogenesis

Over the years, numerous hypotheses on the molecular mechanism of action of retinoids in control of differentiation have been proposed, but none has stood up to the experimental data. In particular, any hypothesis relating to molecular mechanism of action must take into account the evidence, now overwhelming, that retinoic acid will support growth in the whole animal as effectively as retinol (74), that retinoic acid is more active than retinol or retinoid in numerous in vitro test systems (11, 30, 57, 62), and that there is no evidence that the mammalian organism can convert retinoic acid to retinol (19). In many test systems, retinoic acid is at least 100 to 1000 times more active than retinol (11, 57, 62), and biological activity can be measured at levels as low as 10^{-11} M (57). Thus, the hypothesis, proposed in the 1960s, that retinol directly modifies membrane structure (16) to exert its biological effects is now of only historical interest.

More recently, it has been suggested that a primary biological role of the retinoids is to participate in sugar transfer reactions by means of the intermediate retinyl phosphate mannose, which is a metabolite of retinol (1, 15, 71). This hypothesis cannot be rationalized with the experimental data on retinoic acid, summarized above. Neither is there any convincing evidence at present for a metabolite of retinoic acid which is involved in sugar transfer reactions. The recent synthesis of a new series of retinoids (29), which may be viewed as retinoidal benzoic acid derivatives (Chart 1) and which are even more potent than retinoic acid in many test systems both in vivo and in vitro, provides even further experimental evidence against any essential role for retinyl phosphate mannose in control of differentiation or carcinogenesis. The new analogue shown in Chart 1 (or its derivatives) will support growth in the whole animal fed a vitamin A-deficient diet (29), is at least 1000 times more active than retinol in suppressing skin carcinogenesis in the mouse (29), is more than 100 times as active as retinol in the hamster tracheal organ culture system (57), and is more than 1000 times as active as retinol or retinyl acetate in the F9 teratocarcinoma or HL60 promyelocytic leukemia test systems (61). With data such as these at hand, it is unreasonable to believe that retinyl phosphate mannose plays any universally critical role in the control of differentiation or carcinogenesis. Although one cannot exclude the possibility that there may be some situations in which retinyl phosphate mannose may play some role, currently available information would relegate this metabolite to a minor role in control of differentiation and carcinogenesis.

If one wishes to develop a molecular hypothesis of retinoid mechanism that is compatible with the broadest range of experimental data, then the simplest one that can be proposed at present is to suggest that retinoids modify gene expression. This, of course, is not a new idea. If one takes this as a general proposition, then 2 important questions follow: (a) which genes
are controlled by retinoids; and (b) how are these genes controlled by retinoids? (is the mechanism one of direct or indirect control?) We will provide only an outline of what is known regarding these 2 questions.

With respect to which genes are known to be controlled by retinoids, one is impressed by the number of recent reports which indicate that retinoids control the expression of many proteins which either are direct constituents of the cytoskeleton and extracellular matrix or participate in the formation of cytoskeleton and matrix. These proteins include keratins (22), collagen (62, 63), collagenase (12), transglutaminase (53, 73), and laminin (63). Determination of the specific types of cytoskeletal or matrix proteins which are produced in cells is now being used as a specific marker for cell differentiation, and it would appear that retinoids are intimately involved in this process. Other proteins the expression of which is known to be controlled by retinoids include plasminogen activator (62, 63), alkaline phosphatase (48, 63), and the receptor for epidermal growth factor (25, 47). Furthermore, the important observation has recently been made in the HL60 system that retinoid acid controls the expression of the myc oncogene. Using a specific molecular probe for the myc gene, it has been shown that physiological levels of all-trans-retinoic acid suppress myc gene expression in HL60 cells (Ref. 68; Fig. 3). Although the specific molecular function for the myc gene product has not yet been elucidated, it is presumed that in some way the excessive expression of this gene and its product is correlated with the excessive proliferation (and perhaps with the arrested differentiation) of the HL60 cell. However, the possibility must be considered that an oncogene other than myc may also contribute to the neoplastic behavior of the HL60 cell and that retinoic acid may have significant interactions with this gene as well. Furthermore, as of the present, the kinetics of the interaction of retinoic acid with myc in HL60 has not been determined, and it is not yet clear whether retinoic acid controls myc expression directly or indirectly.

Thus, at the gene level, it appears that retinoids affect the expression of genes or gene products involved with both differentiation and proliferation. The remaining, and most difficult, question is, "How do they do it?" The overall problem of the control of gene expression is beyond the scope of this article; (control of gene transcription itself, regulation of translation of primary gene transcripts, or regulation of translation of processed message. Little is known about retinoids in any of these areas. Recent experiments have demonstrated effects of retinoids on genomic expression in retinoid-deficient rat tissues (43); however, the complexity of the experimental system precludes analysis of the molecular mechanisms involved. By analogy with the steroids, it has been suggested that the effects of retinoids in controlling gene expression are mediated by specific intracellular binding proteins (13). However, retinoids have significant effects in control of both differentiation and carcinogenesis in 2 important cell systems, namely HL60 and 10T1/2 fibroblasts, in which no retinoid-binding protein (analogous to steroid-binding proteins) can be detected (17, 28).

The alternative to a steroid-like mechanism for retinoids is to suggest that they control gene expression via interactions with protein kinases, both cyclic AMP-dependent and cyclic AMP independent. Retinoids have been shown to increase cyclic AMP-dependent protein kinase activity in B16 melanoma cells (32), which are highly sensitive to their antiproliferative effects (30), as well as in F9 teratocarcinoma cells which are induced to differentiate by retinoic acid (45). Furthermore, N[O2]-dibutyryl cyclic adenosine 3',5'-monophosphate markedly potentiates the differentiating effects of retinoic acid in both F9 teratocarcinoma cells (63) and HL60 leukemia cells (42). Very recent work has also suggested that a secondary effect of retinoic acid may be the induction in the F9 system of a calcium- and phospholipid-dependent, cyclic AMP-independent protein kinase activity (2, 41, 64) and that some of the interactions between retinoids and phorbol esters may be mediated through this system (2). These latest studies on a calcium-dependent protein kinase system provide an important link between retinoids and calcium, which is now assuming an increasing importance in control of cell proliferation and differentiation (46, 69). Thus, although studies on the interactions between retinoids and the various protein kinase systems of the cell have only recently begun, they have already yielded significant new data which will need to be integrated into an overall hypothesis of mechanism of action.

Ultimately, it would appear that the problem of the molecular mechanism of action of retinoids in control of differentiation and carcinogenesis is converging on one of the central problems in all of biology, namely, the control of gene expression. There may

2 R. A. Weinberg, personal communication.

3 T. R. Breitman, personal communication.

4 The abbreviation used is: cyclic AMP, cyclic adenosine 3',5'-monophosphate.
be new mechanisms, yet to be discovered, that may be critically involved in this process. For example, yet another question that awaits further experimentation is the functional relationship between retinoids and polypeptide growth factors that control cell proliferation and differentiation (58). The role of peptide growth factors in controlling these processes is of fundamental importance (for a review, see Ref. 9); for example, there is evidence that a new family of transforming growth factors (type α and type β), which we have recently described (3, 49), may play a key role in the processes of carcinogenesis and differentiation. Clearly, any future hypothesis dealing with mechanism of action of retinoids will need to integrate the role of retinoids, peptide growth factors, and specific genes controlling differentiation and proliferation. In particular, it must define the precise relationship between retinoids, transforming growth factors, and cellular oncogenes. The breadth, potency, and specificity of retinoids in the control of cell function all suggest that retinoids will be valuable tools for the experimental scientist to unravel molecular mechanisms, in addition to their practical usefulness in controlling carcinogenesis and carcinogenesis.

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References

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Fig. 1. Left, normal 3-day avian embryo. Note well-developed extraembryonic circulatory system, with large major blood vessels surrounding the embryo. Right, abnormal 2-day avian embryo, resulting from retinoid deficiency. Although many structures, such as the somites, have formed relatively normally, there is a conspicuous absence of an extraembryonic circulatory system. Treatment of early embryos with retinoic acid methyl ester will restore normal development. Reprinted with permission from Ref. 66.

Fig. 2. Retinoic acid induces morphological and functional maturation of leukemic cells obtained from a patient with promyelocytic leukemia. A, cells cultured without retinoic acid consisting of promyelocytes with characteristic cytoplasmic granules. × 860. B, cells cultured with retinoic acid showing maturation to banded and segmented neutrophils. × 860. C, absence of nitroblue tetrazolium reduction by cells cultured without retinoic acid. × 315. D, nitroblue tetrazolium reduction by cells incubated with retinoic acid. × 315. Reprinted with permission from Ref. 10.
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