Regulatory Controls of Oncotrophoblast Proteins and Developmental Alkaline Phosphatases in Cancer Cells

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Summary

The two oncotrophoblast proteins, Regan isoenzyme (placental-type alkaline phosphatase) and human chorionic gonadotrophin, are readily studied oncodevelopmental gene products in human cancer patients and in three experimental model systems. The latter consists of (a) HeLa sublines TCRC-1 and TCRC-2, which produce Regan and non-Regan isoenzymes, (b) HEp-2 and FL amnion cell lines as models for the reciprocal expression of developmental genes, and (c) modulation in vivo of developmental gene expression in HeLa cells. In the case of the third model, for example, HeLa TCRC-1 cells grow in immunosuppressed rats to form a tumor nodule, which expresses a new onco-amnion (FL) isoenzyme, while the Regan isoenzyme disappears. Return of the tumor cells to cell culture medium results in a disappearance of the oncoamnion (FL) species and the reappearance of Regan isoenzyme. This interesting model is expected to bridge the interpretation of experiments done in cell culture with observations made on tumors of cancer patients. Most helpful in interpretation of these studies has been a chronology of early development. It appears that the counterparts of a number of tumor proteins appear as early as gametogenesis and as late as 10 weeks of gestation.

Introduction

Two general experiences typify work in the field of oncodevelopmental proteins. First, human tumors are the source of almost all the oncodevelopmental gene products being studied today and, except for a-fetoprotein, the order of discovery of the particular gene product has been tumor first and developmental counterpart second.

Next, although such gene products may be expressed in a wide variety of tumors, yet the nature of the tumors that most frequently produce particular developmental proteins has acquired biological significance. Thus, a-fetoprotein is identified primarily with teratocarcinoma and hepatoma, CEA with cancers of the gastrointestinal tract, and Regan isoenzyme and HCG with cancers of the ovary and testis.

The fact that 2 trophoblast proteins, a term placental-type alkaline phosphatase (Regan isoenzyme) and HCG, also show a degree of concordance of expression (6) in patients with ovarian cancer and testicular carcinoma is a circumstance that becomes intriguing following the recent report by Francois Jacob (Second International Congress on Differentiation, Copenhagen, September 1975, personal communication) that gonadal genes can be reexpressed in early development and in neoplasms (mouse teratocarcinoma).

A stimulus to evaluating developmental gene products in terms of the chronology of development has come from discoveries that many tumors express an alkaline phosphatase isozyme that is indistinguishable from that present in 6- to 10-week trophoblast (3), some hepatomas produce an isozyme identical to the fast alkaline phosphatase of FL amnion cells (17), and a variety of tumors express term placental alkaline phosphatase (Regan isoenzyme). When a chronology of development is constructed (Chart 1) and the position of the oncocional counterpart gene product is marked, it is interesting that most of these are products appearing between 1 and 10 weeks of gestation. It is also interesting that the tumors that produce a-fetoprotein are not known to express trophoblast products except for the embryonal tumors which possess both yolk sac and trophoblast elements. The converse appears to be true also. Does this mean that embryoblast gene sets are on a different circuit of activation than trophoblast gene sets? What is the neoplastic significance of proteins expressed by both placental and fetal genes?

If we can agree that current research with regard to the questions raised above can only be considered exploratory, then we can be comfortable in presenting some observations which have been provocative at least to us.

This paper deals specifically with oncotrophoblast proteins, not only because we are most familiar with them, but because the expression of these gene products may identify those tumors that acquire the biological characteristics of trophoblast which in turn could endow the cancer cell with invasiveness and autonomy. It also describes the cancer cell model systems in which it is possible to observe modulation of developmental gene expression, observations that have their counterparts in human cancer tissues.

Results

Chorionic, Testis, and Teratocarcinoma Alkaline Phosphatase Isoenzymes. The interesting characteristic of early trophoblast alkaline phosphatase is the presence of a fast-
Regulation of Oncotrophoblastic Proteins in Cancer Cells

EARLY HUMAN DEVELOPMENT

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Chart 1. Chronology of development of oncological gene products. AFP, α-fetoprotein; CAP, chorionic alkaline phosphatase; PAP, placental alkaline phosphatase.

ment gene products. Their properties are summarized in Table 1.

With particular reference to alkaline phosphatase, use of each of these 2 cell lines provides information that is more accurately interpreted than the results obtained with wild-type HeLa cells which produce a mixture of the Regan isoenzyme (term placental alkaline phosphatase) and non-placental enzyme. In such HeLa cells, isoenzyme analysis is necessary because a change in total enzyme activity may result from an event that enhances the activity of Regan isoenzyme while depressing the non-Regan isoenzyme activity.

The non-Regan isoenzyme is now considered to be chorionic or early placental in type (3). It is not inducible by prednisolone in the medium, suggesting a process of gene regulation different from that functioning in HeLa TCRC-1 cells.

HEp-2 and FL Amnion Cell Lines as Models for the Reciprocal Expression of Developmental Genes. Both of these cell lines under appropriate experimental conditions express Regan isoenzyme and another isoenzyme (on-

moving band that lacks antigenic determinants known for liver, bone, and intestinal alkaline phosphatases (3). A slower band shares antigenic determinants with the liver isoenzyme. Both bands are heat sensitive and are inhibited by L-homoarginine but not by L-phenylalanine.

As a consequence of Jacob's observations, we were motivated to study the isozymes of human testis (Fig. 1). Much of the activity is heat sensitive and fails to react with antisera to liver, intestine, and placental alkaline phosphatase. Following heat inactivation (5 min at 65°), the electrophoresis revealed heat-stable bands, one of which cross-reacted with antisera to placental alkaline phosphatase. This mixture of alkaline phosphatase is relatively insensitive to L-phenylalanine, inhibited by L-homoarginine, and 80% inactivated by heat (3).

Next, a specimen of testicular teratocarcinoma was homogenized, and the alkaline phosphatases of unheated and heated extracts migrated out on cellulose acetate membranes (Fig. 2). Here, isozymes similar to testis were observed, a heat-sensitive one lacking antigenic determinants known for tissue alkaline phosphatases and a heat-stable one possessing the antigenic determinants of term placental alkaline phosphatase. There is also a suggestion of intestinal alkaline phosphatase, since some isozyme retardation occurred with antisera to intestinal alkaline phosphatase.

Although many more specimens must be studied, the data do provoke the thought that the trophoblast alkaline phosphatases may be expressions of testicular genes. It would not be surprising if testicular teratocarcinoma cells expressed testicular genes. However, there is evidence that an isoenzyme lacking the antigenic determinants of liver, intestine, and placenta is expressed in undifferentiated bronchogenic cancer (7).

HeLa Sublines TCRC-1 and TCRC-2 as Models for the Study of Developmental Gene Expression. Two clones of wild-type HeLa cells have been established (16) and have proven to be valuable in the study of regulation of develop-

Fig. 1. Microzone electrophoresis of human testis demonstrating retardation by antisera to intestinal (I), liver (L), and placental (P) alkaline phosphatase. Antibody (3) (Ab) retardation is also shown for heated samples.

Unheated 5' at 65°C

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Fig. 2. Microzone electrophoresis of human teratocarcinoma, characterized as described in Fig. 1. Ab, antibody; I, intestinal; L, liver; P, placental.
coamnion FL) which is fast moving on acrylamide gel electrophoresis, is partially heat inactivated, is inhibited by L-leucine, and cross-reacts with antiserum to intestinal alkaline phosphatase (17). This isoenzyme is indistinguishable from the human hepatoma variant of Warnock and Reisman (23).

Prednisolone in the medium produces an interesting modulation in HEp-2 cells of these 2 isoenzymes; the Regan isoenzyme undergoes an enhancement in activity, while the oncoamnion isoenzyme is reduced (8) (Chart 2). Similar results were obtained by hypertonic salt solution (8).

**Cell Cycle Events in the Expression of Alkaline Phosphatase in HeLa TCRC-1 in Vitro.** Published experiments have demonstrated (19) both that prednisolone blocks TCRC-1 cells in G1, at which juncture they produce Regan isoenzyme, and that no alkaline phosphatase synthesis is initiated in the S period (Chart 3).

With regard to oncofetal proteins, the studies of Sell et al. (14) and of Tsukada and Hirai (22) have shown that, in rat liver cells, α-fetoprotein initiation occurs at the G1-S boundary. Interestingly, Belanger et al. (1) have stimulated α-fetoprotein synthesis in hepatocytes in G1 with prednisolone.

Opportunities for the study of expression of gene products in G1 may determine whether prednisolone induces α-fetoprotein and trophoblast proteins at the same or different positions in the cell cycle.

**Modulation in Vivo of Developmental Gene Expression of HeLa cells.** In an attempt to examine the biological significance of oncodevelopmental isoenzymes of alkaline phosphatase in human neoplasia, we have developed a model system that permits the modulation of these isoenzymes in vivo (15, 18). Thus, HeLa TCRC-1 and TCRC-2 cells were transplanted s.c. into neonatally immunosuppressed Wistar-Lewis rats according to the method of Sorvari and Arvilommi (20). They grew as solid tumor nodules (18). A dramatic alteration was observed in the isoenzyme profile during growth in vivo. In Chart 4, the heat stability of alkaline phosphatase from TCRC-1 and TCRC-2 is measured during growth in immunosuppressed rats. It can be seen that the heat-stable Regan isoenzyme from HeLa TCRC-1 disappears and a partially heat stable enzyme form appears. HeLa TCRC-2 alkaline phosphatase, which is totally heat labile, is replaced by an enzyme that has similar heat-stability characteristics of the final enzyme form produced by HeLa TCRC-1 growing in animals. This biochemical conver-

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**Table 1**

| Contrasting biological and biochemical properties of 2 sublines of HeLa cells |
|--------------------------|----------------|
|                         | TCRC-1 | TCRC-2 |
| **Alkaline phosphatase** |         |        |
| Kinetic properties       |         |        |
| Specific activity (μmoles/min/μg) | 0.75   | 3.2    |
| L-Phenylalanine inhibition (%) | 73.1   | 0.0    |
| L-Homoarginine inhibition | 11.5   | 77.5   |
| Heat inactivation (%, 5 min at 65°) | 10.9   | 100.0 |
| **Immunological characteristics** |         |        |
| Placental determinants   | +      | -      |
| Intestinal determinants  | -      | -      |
| Liver determinants       | -      | +      |
| Induction                |         |        |
| Prednisolone effect on specific activity (%) | +175   | 0      |
| **Acidic isoferriptins** |         |        |
| Induction by inorganic iron | +     | -      |
| β-Glucuronidase          | +      | +      |
| Induction by prednisolone | +      | -      |
| Growth in immunosuppressed rats | +      | +      |

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**Chart 2.** Gel scan of isoenzymes of alkaline phosphatase in FL amnion cells. Prednisolone (PR) and osmolarity (NaCl) are compared to the control untreated culture (8).

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**Chart 3.** Cell cycle model system for prednisolone effects on cell cycle (19). PR, prednisolone; HU, hydroxyurea.
sion suggests an adaptation phenomenon that may result in a cell type most able to form and maintain the growth of a tumor nodule in vivo.

Upon closer examination of the isoenzyme alteration in these cell lines with the use of acrylamide gel electrophoresis, a shift is seen in the isoenzyme components of these cells growing in immunosuppressed rats. As shown in Chart 5, the HeLa TCRC-1 Regan isoenzyme band is absent after 30 days in vivo, and an enzyme form with all the properties of the oncoamnion (FL) isoenzyme is present. It has many of the properties of the adult human intestinal alkaline phosphatase; however, it differs in that it is inhibited by L-leucine and is partially heat stable. During the shift from the Regan to the oncoamnion (FL) isoenzyme, there is a transient chorion-like enzyme form (3) which is present at 20 days of tumor growth. It was found to be identical in all respects to that found in the TCRC-2 cell line and early placenta (3). It can also be seen that, when HeLa TCRC-2 is grown in animals, this cell line also produces an isoenzyme that has all the biochemical and immunological characteristics of the oncoamnion (FL) isoenzyme. However, the constitutive chorion isoenzyme of TCRC-2 does not disappear and is present throughout the course of tumor growth in vivo, and Regan isoenzyme does not appear.

It is especially noteworthy that, after HeLa TCRC-1 cells are passaged through an animal and returned to culture, they differ from the original cell line with respect to their isoenzyme profile. During the early stages of growth in culture, these cells produce the Regan isoenzyme, while in the later stages, when cell density is greatly increased, the oncoamnion isoenzyme is reexpressed (15). This alteration in isoenzymes in culture appears to be related to the stage of growth of the culture, or to the density of the cells in the culture flask, or both.

It is also interesting that when the slowly migrating Regan isoenzyme is replaced by the faster-moving oncoamnion (FL) enzyme form, a transient enzyme species is always observed, with a migration distance midway between these isoenzymes. This intermediate enzyme form shares many of the properties of the Regan and oncoamnion (FL) band, and may be due to a hybrid molecule containing subunits from both of these isoenzymes.

Discussion

The HeLa model system we have chosen to study has been used by other investigators to study the regulation of other trophoblast gene products found in cancer. For example, HCG, which is (like placental alkaline phosphatase) a product of the syncytiotrophoblast, is found in elevated levels in neoplastic and preneoplastic tissues (7). Both the α subunit (12) and β subunits (9) of this polypeptide hormone have been shown to be produced by HeLa cells. Prednisolone, which greatly elevates the Regan isoenzyme in HeLa cells, increased the levels of HCG-β only slightly (9). Sodium butyrate, however, which has also been shown to be an inducer of alkaline phosphatase (9), caused a marked elevation in HCG-β levels. Thus HCG-β is another trophoblast gene product which is expressed in HeLa cells and whose expression is inducible by appropriate treatment, sodium butyrate inducing both HCG-β and Regan isoenzyme, while prednisolone induces only the latter. Thus, the list of trophoblast gene products in HeLa cells now includes early chorionic as well as the Regan isoenzyme of alkaline phosphatase, and HCG (α and β subunits) and a number of specific inducers. (It should be mentioned that the tumor homogenate of the original Regan isoenzyme exhibited significant amounts of HCG (unpublished data).]

In the general category of oncodevelopmental gene expression, HeLa cells have also been shown to produce carcinoembryonic acidic isoferritins (2), as well as an elevated level of β-glucuronidase (16). Thus, our present HeLa cell studies on the modulation of oncodevelopmental isoenzymes of alkaline phosphatase by hormones and in vivo growth will be expanded to include all...
the modulation of the known cancer-related gene products. For example, we shall determine whether the specific induction of the Regan isoenzyme by prednisolone in culture is an operational regulatory mechanism in these cells while growing in an animal host. The induction by iron of carcino-fetal isoferritin, as well as HCG-β product production and induction, in HeLa cells growing in animals may shed more light on the significance of these oncodevelopmental antigens produced by tumors growing in syngeneic human hosts. In all these cases, attention is being paid to cell-to-cell interactions and the role of the cell cycle in gene expression.

Are there counterparts in human tumors of the coexpression of oncodevelopmental phosphatases? This laboratory has reported the presence of both Regan and non-Regan isoenzymes in ovarian cancer and a degree of concordance of expression of these isoenzymes with HCG in a number of longitudinal studies (6). More recently, Suzuki et al. (21) have shown that, of separate extracts of gastric cancer tissue, 4 of 11 exhibited Regan and non-Regan isoenzyme, and 2 of these 4 evidence intestinal alkaline phosphatase. The others presented only a "liver-type" isozyme. If this intestinal component turns out to be oncoamnion (FL), and the liver type, chorion, then this assembly of isoenzymes may reflect the same phenomenon described here in the xenograft experiments as modulation in vivo. "Intestinalization" is a term that is acquiring biological significance in many varieties of cancer such as colon, ovary, and gallbladder (4). Cell-to-cell interactions may be important also in the eventual explanation.

Chronology of Early Human Development and Gene Expression in Tumors. The recognition that all of the human tumor phosphatases have their counterparts at specific events or in tissue lineages during development has motivated us to construct a chronology of development as a reference point. Chart 1, with an expanded scale for the 1st 3 weeks, presents gametogenesis as the first event and fertilization producing the zygote as the next. Proceeding through the stages of morula and blastocyst, by the end of the 1st week, implantation takes place; the blastocyst at this time consists of an inner cell mass, embryoblast, and an outer layer of cells, tropheoblast, which make entry through the uterine mucosa. Shortly afterward, one can recognize the extraembryonic mesoderm that invests tropheoblast, amnion, and yolk sac (entoderm). It is seen that tropheoblast cells give rise to cytotrophoblast, which differentiates into syncytiotrophoblast cells. The latter develop from primary through secondary and tertiary stem villi that form the chorion frondosum, whereas the chorion laeve is a membrane (chorion) that fuses with the amnion membranes to form the placenta at 12 weeks.

The embryo itself exists from 4 to 8 weeks of gestation as a product first of the 2-germ layer (entoderm and ectoderm) and then of the 3-germ layer (entoderm, ectoderm, and mesoderm) preembryonic tissues. After organogenesis during this period, the fetus is the proper word to describe the emerging nonembryonic organism.

Exclusively oncotrophoblast products are HCG at 1 week, chorionic alkaline phosphatase at 6 weeks, and placental alkaline phosphatase at 10 weeks. The latter 2 products may represent reexpression of testicular genes, as suggested earlier.

Exclusively embryoblast gene products in tumors are α-fetoprotein in yolk sac at 3 weeks and in fetal liver from 4 weeks on. CEA was first reported in 2- to 4-month-old fetuses by Gold and Freedman (10). It is expected that CEA will be identified with earlier developmental dates in more detailed studies.

Those products, such as the acidic isoferritins and pyruvate kinase, which are found in both tropheoblast and embryoblast lineages, suggest the possibility that they may derive from extraembryonic mesoderm which invests both systems. Angiogenetic cell clusters may be the sites of synthesis of these proteins.

References

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