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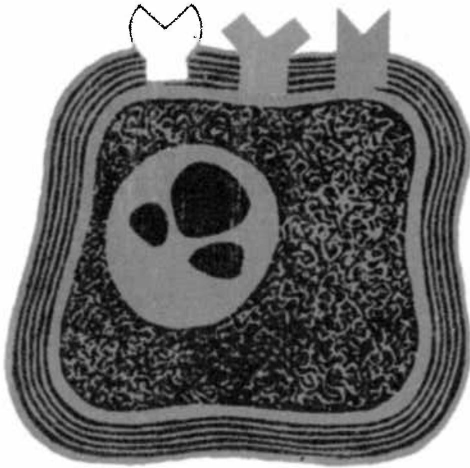

Journal of Tumor Marker Oncology

the official journal of the International Academy of Tumor Marker Oncology

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The study model that we used for cell differentiation is the Zebrafish embryo, as it features regulators that are maintained during phylogeny.

This simplifies research significantly and does not pose insurmountable ethical problems.

We trust that the publication of the following results may prompt other work groups to continue these studies. The fight against cancer is difficult and long: it involves 130 different diseases, each of which requires a specific regulation therapy. We have blazed a new trail that we trust will be further explored by others. If this happens, credit should be given to the role played by the Editorial Committee of the Journal of Tumor Marker Oncology that actively brings this research work to the attention of the international community. My thanks therefore go to Professor Klavins, Professor Birkmayer and the other members of the Journal's Editorial Committee for their keen interest in our research findings.

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Cancer and Cell Differentiation: A Model to Explain Malignancy

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Introduction

The evidence obtained from studying the interactions between tumor cells and embryonic tissues suggests that tumor development in embryos is reduced or suppressed when differentiation processes are in progress (1,2).

The administration of known carcinogens during cell differentiation in embryos causes malformations in offspring, but not tumor induction. Once organogenesis is complete, the frequency of tumor induction rises concurrently with the decrease in the rate of malformations (3,4,5).

These findings could indicate that cancer can be viewed as a developmental deviation that may be controlled by cell differentiation regulators.

Based on the foregoing, experiments on animals were carried out. Past experiments have demonstrated that factors present during cell differentiation can stop or delay tumor growth in animals. These factors are present in the pregnant uteri of mammals (6) and in the embryos of ovipara (7). More recent experiments in vitro have shown that pregnant pig and mouse uterus extracts slow down the proliferation rate of several established human tumor cell lines (8). It was clarified that the abnormal growth of cell clones during embryo organogenesis in mammals is prevented by low molecular weight substances present in the pregnant uterus microenvironment. A 5kDa fraction isolated in our laboratory from the pregnant uterus of mammals, named "Life- Protecting Factor", inhibited the cell proliferation curves of all treated human tumor cell lines as well as the crude pregnant uterus extracts. Therefore, the interactions between mother and embryo seem to be important for the normal development of the embryo and for preventing pathological cell growth. The embryo itself seems to prevent the abnormal multiplication of tumor cells. In fact it was demonstrated that different tumor cell lines responded with a significant slowdown in proliferation when treated with extracts taken during the stages of cell differentiation, while no slowing effect was observed when they were treated with the extracts taken from a merely multiplicative stage (9). Thus, cell differentiation is a key process in understanding the behavior of both normal and tumor cells. The fact that embryonic development and tumorigenesis are closely correlated is now accepted: they both share several pathways and molecules that can regulate some important genes of the cell cycle. The main effect of the in vitro treatment of tumor cell lines with the extracts of oviparous embryos is the activation of p53

expression, as observed by immunohistochemical and flow cytometry techniques after treating different tumor cell lines with fish embryo extracts (10). In addition, in another article of this issue, we have reported the induction of a post-translational regulation of pRb by zebrafish embryonic extracts, which is probably responsible for the observed slowdown of kidney adenocarcinoma proliferation curves in vitro. Embryonic differentiation and tumorigenesis, although they share several metabolic pathways seem to be opposite processes: the same molecules, which cause cell differentiation in embryos, seem to be able to inhibit cancer growth. In order to explain the mechanisms involved in these two different processes, it is necessary to illustrate an outline and a model of embryonic differentiation and cancerogenesis.

An outline and a model of embryonic differentiation

The differentiation processes begin shortly after fertilization, generally in the middle-blastula-gastrula period. There are three postulates of cell differentiation:

1. every cell nucleus contains the complete genome established in the fertilized egg. In molecular terms the DNAs of all differentiated cells are identical;
2. the unused genes in the differentiated cells are not destroyed or mutated and they retain the potential for being expressed;
3. only a small percentage of the genome is expressed in each differentiated cell and a portion of the synthesized RNA is specific for that cell type.

Briefly, the differentiation, which leads pluripotent embryonic stem cells to specialization, consists in a differential regulation of genes that restricts the expressed genome. The gene configurations of the cells after each stage of differentiation differ from the progenitors for some thousands of expressed genes.

Regulators are generally factors that cooperate in a network and this network promotes and controls the differentiation of each cell type. All cells communicate with each other through this network.

Cell differentiation is a very complex process that takes place at different levels:

A) a differential gene transcription which regulates how the nuclear genes are transcripted into RNA;
B) a selective nuclear RNA processing which regulates how the transcript RNA's get into the cytoplasm to become messenger RNA's;
C) a selective messenger RNA translation that regulates how messenger RNA's in the cytoplasm is translated into proteins;
D) a differential modification of proteins, which regulates how proteins are allowed to function in the cells.

Transcription factors are very important in controlling the differential expression of genes, but in eukariotes selective nuclear RNA's processes are more important. These selective processes clarify how the same gene can produce two different proteins in different cells or in the same cell at different times.

Moreover selective degradations or, otherwise, selective stabilizations of the messenger RNAs are responsible for further protein specifications.

Today we have a dynamic vision of the regulation of gene expression.

A gene is not thought to be an independent and autonomous control center of protein synthesis.

A gene is also controlled directly or indirectly by the synthesized proteins.

The interactions between nucleus and cytoplasm and between cytoplasm and microenvironment are so extensive that they constitute a marvelous example of complexity.

The developing embryo is an excellent example of "complex adaptive systems".

In fact the embryo is, 1) a network of many cells acting in parallel, 2) has many levels of a constantly revising and rearranging organization, 3) has an implicit prediction encoded in its genes and 4) is always in transition and is characterized by ongoing innovations.

Cell differentiation can be better understood in a model described here, which is consistent with the real situation. In this model the number of final gene configurations of cells in the human body (number of types of completely differentiated cells) can be predicted, if we retain that each kind of progenitor cell produces 3 different daughter cells (3 different "gene configurations") and that there are 5 stages of differentiation (fig. 1).

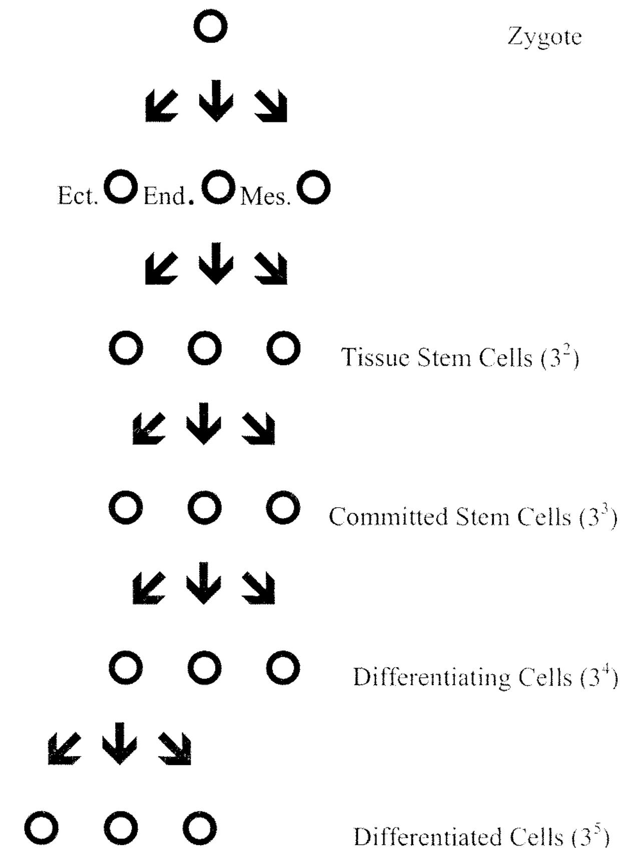

Fig. 1: Model of Cell Differentiation

This corresponds to the real situation: in fact the embryo, after segmentation (morula), is differentiating in three layers: ectoderm, endoderm and mesoderm. Gametes are differentiating in a different pathway compared with somatic cells. After gastrulation, there are another four stages of cell differentiation. For example, on the basis of precise data about some cell lines, such as hematopoietic cells, the stages of differentiation are: a) stem cell stage, b) committed stem cell stage, c) differentiating cell stage, d) differentiated cell stage. If we include the ectodermal, endodermal and mesodermal cell lines, there are five stages of differentiation. Therefore the mathematical formula to calculate the number of differentiated cells is:

$$N = 3^5.$$

The result is 243, which is the number of the different somatic differentiated cells. To calculate the final number of the differentiated cells we must add the number of gametes. The sex cells are 5 in males (spermatogonium, spermatocyte of the first order, spermatocyte of the second order, spermatid, spermatozoon) and 4 in females (ovogonium, ovocyte of the first order, ovocyte of the second order, egg cell).The final result is 252, which is the number of the different kinds of cells effectively counted in humans. Life is organized through very simple algorithm!

Cancer as undifferentiated mutated cells. A model to explain malignancy

The tumoral transformation of normal cells is a process with a minimal number of stochastic mutational events, between 4 and 7 (11). If mutations are introduced into normal cells in a non-stochastic manner, i.e., triggering at precise genes, the number is reduced (12). The preferred targets of these mutations are genes encoding for key-role effectors of cell cycle regulation and cell signaling, and for growth factors and their receptors; mutations are either gain-of-function, in the case of proto-oncogenes, or loss-of-function, in the case of tumor suppressor genes.

Defining the tumoral transformation of a cell simply as the outcome of a sum of gene mutations may be restrictive. For normal cells to become cancerous, transformation also depends on a complex network of surrounding microenvironmental signals from cell-to-cell "cross-talking" or from soluble extracellular factors. For example, it has been demonstrated that fibroblasts adjacent to prostate epithelium carcinoma cells are capable of directing tumor progression (13), that stromal neighbor cells are capable of promoting malignant transformation of immortalized keratinocytes by releasing proliferative stimuli (14), and that inflammatory cells can sustain, instead of fight, tumor growth (15). Even proinflammatory cytokines were shown to promote cancer cell proliferation by inhibiting tumor suppression pathways (16). Thus, the whole context is decisive in determining cell fate in line with a "heterotypic" view of cell biology, as it was called in a recent review (17). According to this view, defining tumorigenesis as a microevolutive process is now safe. A cancer cell acquires, as a consequence of this process, some capabilities: 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) the ability to evade apoptosis, 4) limitless replicative potential, 5) the ability to sustain angiogenesis, 6) the ability to invade tissues and give metastasis. The acquisition of these capabilities during the course of tumor progression is usually the consequence of a great variability of the mechanisms used by cells to become malignant. Nonetheless the hypothesis presented here is that regardless of how the steps in these genetic pathways are arranged, the development of all types of human tumor cells is governed by a final common process. Some authors define "early crisis" and "genetic catastrophe" of cells some of the steps that enable the evolving population of premalignant cells to reach malignancy (18). As a result of these crises, during which a telomere dysfunction and DNA damage take place: 1) cells die or 2) cells survive after each crisis. The final results are adaptive responses and telomere maintenance in the case of cell survival. Surviving malignant cells have A) not only increased the level of telomerase, but B) have also activated proto-oncogenes or oncogenes, C) produce growth factors, D) are insensitive to anti-growth signals, E) have several surface antigens, also known as oncofetal antigens, maintained during phylogeny (19), most of which have been identified in the last 30 years (20-30). In other terms the cells that survive a period of genetic

instability become malignant through the achievement of a new stable gene configuration very similar to those present in the embryo during periods of multiplication. Cancer cells and embryonic cells share some molecular pathways and their key-role effectors: e.g. the APC/ β-catenin/ TCF/ Wnt pathway (31,32) and the Hedgehog/Smoothened/Patched pathway (33). In embryonic development these pathways lead cells to successful differentiation, in tumorigenesis their mutated counterparts lead cells to constant multiplication. This happens because a cancer cell is an undifferentiated cell in which the mutations present in its genome do not allow the cell to complete the whole program of differentiation and development. It is stopped in a step of the multiplication process, comprised between two stages of differentiation. Figure 2 shows the development of an embryonic cell line.

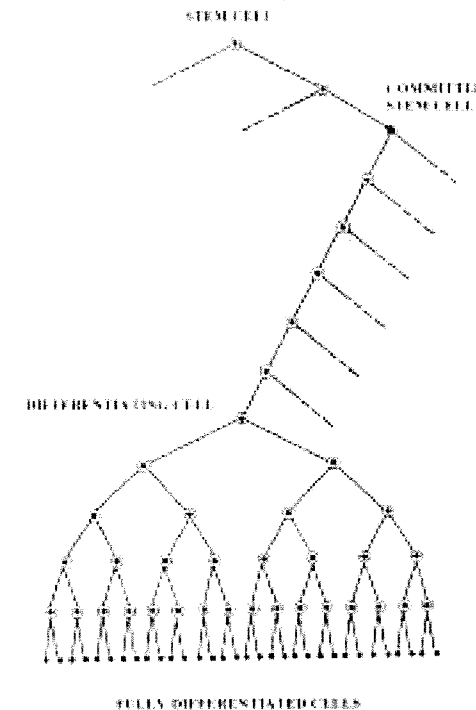

Figure 2: Schematic Model of Differentiation of a Non-Specified Cell Type starting from its progenitor cell

It is possible to see that there are some steps of multiplication between two stages of differentiation. A cancer cell can be defined as an "undifferentiated mutated cell", in which the differentiation and multiplication programs are uncoupled. It is like a computer in loop, repeating always the same instructions. Cancer is probably an example of deterministic chaos. It is a branching process, that leads the cell, since it does not die, to rampant genetic instability: the final attractor is a new stable "gene configuration" similar to that present in the embryo during the steps of multiplication, between two stages of differentiation (fig. 3). In line with this, considering the model of cell differentiation previously mentioned, the number of different types of cancer deriving from somatic cells can be predicted with the formula:

$$N= 3 + 3^2 + 3^3 + 3^4 = 120$$

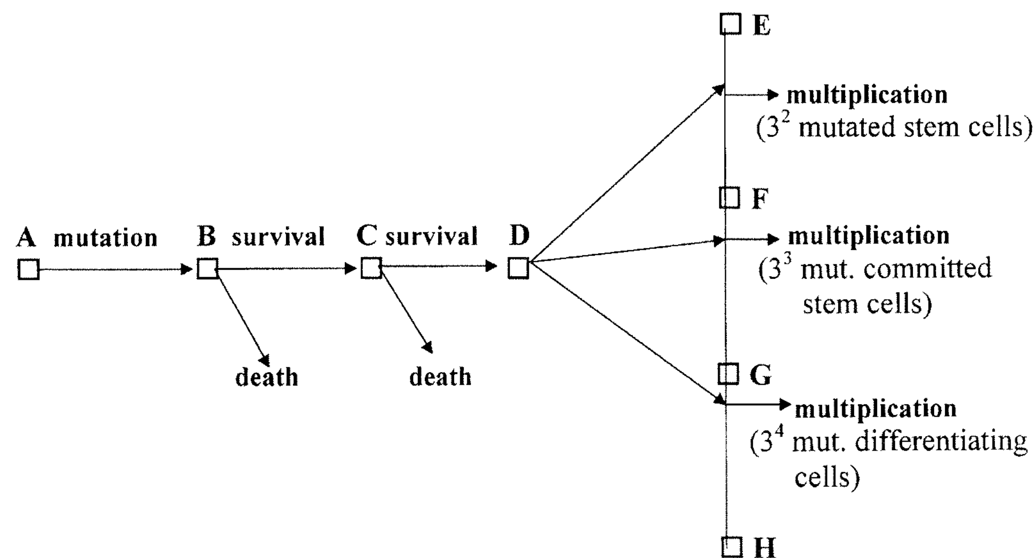

Figure 3: Cancer as a process of deterministic chaos. A: normal cell; B: cell with damaged DNA; C: cell with rampant genetic instability, D: cancer cell: stable gene configuration with uncoupled steps of cell multiplication and differentiation. E: stem cell; F: committed stem cell; G: differentiating cell, H: differentiated cell.

In order to calculate the final number of different kinds of tumors, it is necessary to add the number of tumors coming from sex cells and from different embryonic tissues (teratocarcinoma, embryonic carcinoma, corioncarcinoma). Therefore, the final amount of all different kinds of tumor is about 130. With regard to malignancy, the most aggressive tumors are represented by cells with "gene configurations" present at the early stages of differentiation, that carry out the multiplication program at an impressive speed. The current classification of tumors is redundant because it does not consider that the most malignant types of tumors come from cells that have the same "gene configurations". Finally, some types of tumors come from different cell clones with "gene configurations" arising from different stages of differentiation.

The regulation of cancer growth: a model of complexity

The cancer model above is not merely theoretical, but is based on the results of laboratory experiments. These experiments have shown that molecular factors present during precise stages of cell differentiation are able to inhibit tumor growth. This was demonstrated both *in vivo* on Lewis Lung carcinoma and *in vitro* on several human tumor cell lines. On the contrary, substances present during merely proliferative stages are ineffective in delaying the growth curves of several types of tumors. Thus, cell differentiation is a key-process in explaining the behavior of both undifferentiated normal and tumor cells. Cell differentiation mechanisms are based on a multigenic regulation, so that a more differentiated cell differs from a less differentiated one because of the expression of a great number of genes. Furthermore, according to the model, tumor cells have lost an important portion of the program of cell differentiation in a progressive manner.

Therefore, if the ultimate goal is not to destroy the tumor cell, but its regulation, this can clearly be achieved only by providing the cell with all the factors that can bring it to differentiation. These factors can be found, but only when life is forming. In fact, during organogenesis the whole repertoire of regulatory molecules is present, which includes 1) DNA trascriptional factors; 2) nuclear RNA selection factors; 3) mRNA translational factors; 4) post-translational protein regulatory factors. We have seen that these factors can be used for the genic regulation of tumor cells. A p53-mediated transcriptional regulation and a pRb post-translational regulation were demonstrated, depending on the type of tumor. Thus, it was demonstrated that it is possible to regulate tumor cells, bypassing the mutations that give rise to malignancy. This happens only when the network of differentiation is complete enough. As a result, the focus should be on the microenvironment and networks of the biological structures, rather than on the single subjects of punctual mechanisms. This does not mean that research into molecular mechanisms should be disregarded, but that the single partial mechanisms should be put in a more integrated vision of the biological processes. Indeed, the difficulty in bridging the gap to a new scientific paradigm, that is, shifting our views from reductionism to complexity, has been the main barrier to acquiring a deeper and more complete knowledge of cancer. Studies and researches on stem cell differentiation are proceeding worldwide and the scientific community is ready to accept a new paradigm. These studies will be able to show that differentiation mechanisms are based on specific differentiative networks. The embryonic microenvironment during precise stages of development is fundamental not only for the differentiation of normal stem cells, but also for the differentiation of tumor cells. Embryo, during organogenesis, is never affected by carcinogenetic processes because, while the life program is under transcription, systems of correction in case of mutations are also active. In fact, it has been demonstrated that during cell differentiation, the administration of known carcinogens fails to induce the growth of tumors, perhaps because the genome control system is always working.

According to recent studies, the p53 function in embryo is to prevent malformations, and some authors have called p53 the "guardian of babies", as a gene that suppresses the onset of malformations (34, 35). However, when there is too much stress and mutations are too numerous, the p53 is no longer able to repair the DNA and causes apoptosis in all cells (abortion). These processes also occur in tumor cells when the p53 is activated. This happens when tumor cells are put in contact with embryonic differentiating factors.

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Mother-Embryo Cross-Talk:The Anti-Cancer Substances Produced by Mother and Embryo During Cell Differentiation. A Review of Experimental Data

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The drawing of a new model based on the rather strict links between embryonic and cancer cells as described in the previous review, is based on a series of experimental evidence gathered during the last 15 years. In this review, the *in vitro* and *in vivo* results of all our research work are shown, covering several experimental approaches such as tests on animal models, cytotoxicity assays, and immunohistochemical, flow cytometry and molecular biology methods. The importance of stem cells for our future research is also presented as an intriguing perspective.

Introduction

During pregnancy, the close cross-talk that is formed between the mother and the developing embryo is made of a complex network of molecular factors. This cross-talk is necessary for the prevention of pregnancy-threatening events, including the establishment of abnormally proliferating cell clones, which may damage the integrity of the embryo. This problem had already been encountered by other groups, which referred to activated T-cell clones across the mother-embryo interface (1,2). In the previous review of this, a model that links embryonic development to carcinogenesis, i.e., the onset of an abnormal proliferative program, was described. According to that model, a cell which survives a series of stochastic mutational events finally evolves into a "stable" gene configuration which leads it to develop a cancerous phenotype. These gene configurations can be quantified in a simple mathematical model, and their number is similar to that of known tumoral types. It is suggested that gene configurations of tumor cells correspond to those of normal embryonic cells, but in tumor cells the multiplication and differentiation programs are uncoupled. A delay of tumor cell proliferation may be achieved by providing tumor cells with the complex network of molecular factors that keep those programs coupled in embryonic development.

The development of a theoretical model of the gene regulation of tumor cells by factors of the embryonic microenvironment comes after several years of work, which involved *in vitro* and *in vivo* approaches. In this short review, the results of this work are summarized and shown, giving the reader the experimental bases of the theory presented in the previous paper.

In Vitro Results

In order to assess the effect of the administration of embryonic and decidual extracts on cell proliferation, several tumor cell lines of different origins were used. Zebrafish embryo extracts taken during precise stages of development, i.e., 1000 blastomeres (a full cleavage stage), 50% epiboly (corresponding to the onset of gastrulation), 5 somites and 20 somites were administered to glioblastoma, melanoma, kidney adenocarcinoma, breast carcinoma and lymphoblastic leukemia cells, and proliferation curves were drawn 24 and 48 hours after treatment (3). All cell lines exhibited a slowing down of their proliferation values when treated with extracts from differentiative developmental stages, i.e., from 50% epiboly on. Each cell line had a special slow-down rate and showed a specific response to treatment based on the different developmental stages: for example, the proliferation of glioblastoma was inhibited most by the 50% epiboly stage (named stage I) and least by the 20 somites stage (named stage III), whereas melanoma cells were slowed most by the 5 somites stage (named stage II) and kidney adenocarcinoma cells by stage III. Glioblastoma, melanoma, breast carcinoma and lymphoblastic leukemia cells responded significantly to treatment after 24 hours, whereas kidney adenocarcinoma cells did not exhibit any slowdown at all. After 48 hours however, all cell lines were affected by the treatment, with inhibition percentage values ranging from 73% of the glioblastoma cells and 26% of the melanoma cells treated with stage I extract.

No slowdown response was shown by cells treated with an extract taken from a developmental stage prior to 50% epiboly, namely, the 1000 blastomeres stage (called stage 1k) (3). On the contrary, treated cells exhibited a weak proliferative response. This evidence reinforces our view that differentiative stages of development are characterized by regulatory networks which re-direct tumor cells to a normalized path of differentiation, and that these networks appear from the onset of gastrulation. Before gastrulation, embryos are subjected to merely proliferative stimulating networks which fail to normalize tumor cells, possibly enhancing their abnormal growing potential.

A similar slowing effect on cell growth was observed after the administration of extracts of crude pregnant uterine mucosa to the same tumor cell lines (4). Cells treated with uterine extracts taken from 23 day-pregnant pig exhibited slower proliferation curves, with inhibition percentage values ranging from 80% of the breast carcinoma cells and 67% of the lymphoblastic leukemia cells, to 22% of the glioblastoma cells. As previously observed with zebrafish embryo extracts, each cell line responded to treatment with uterine extracts in a special manner. The day of pregnancy at which the mucosa was collected does not seem to affect the response of tumor cells, since the treatment of glioblastoma cells with uterine extracts taken from pregnant mice on different days led to a diffuse slowing down of the proliferation curve, with inhibition percentage values not significantly different from each other. Finally, the effect of uterine extracts on cell growth appears to be ascribed to tumor cell lines only, since the treatment of a non-tumoral line, murine fibroblast NIH 3T3, did not change the cell proliferation rate.

In order to elucidate which factors are responsible for this effect, we fractionated the whole uterine extract from pregnant pig by low molecular weight cutoffs, and finally we isolated a 5 kDa fraction which retained the slowing efficacy on tumor cell growth. We called this fraction "Life-Protecting Factor" (LPF), because it may contain the molecular factors involved in preserving embryo-mother integrity from pathological cell clones. It is likely that the mechanism of action is apoptosis-mediated, since we observed high levels of a nucleosomal fraction in the medium of tumor cells after 24 hour treatment with uterine extract.

Possible molecular bases of this proliferation-slowing mechanism on cell lines were also investigated by several techniques. Flow cytometry analysis revealed a mean 20% increase of the expression of tumor suppressor p53 in glioblastoma and melanoma cells after treatment with zebrafish embryo extracts (5). Immunohistochemical analysis on treated melanoma and hepatocarcinoma cells showed a dramatic increase of p53 staining compared with untreated cells (5). Not all embryonic extracts were able to induce p53 overexpression, confirming that only precise differentiation stages have the tumor growth-slowing potential. Along with p53, another key-role effector of cell cycle homeostasis, pRb, was shown to be affected by treatment with embryonic factors via the alteration of its phosphorylation state.

In vivo Results

The effect of embryonic factors on tumor growth was also observed in vivo by s.c. injection of primary Lewis lung carcinoma cells into C57BL/6J female mice (6). Immediately before injection, tumor cells were challenged with homogenates of 9 day-pregnant mice uteri, non-pregnant uteri, 9 day-old embryos or liver (the latter being a negative control). Only cells that were mixed with the homogenate of pregnant uteri failed to originate primary tumors: the growth rate was nil for the overall experiment time, whereas cells that were mixed with the other homogenates gave rise to primary masses at the same rate as the unmixed cells. Mice injected with pregnant uterus homogenate-treated cells did not develop spontaneous lung metastases at all, whereas all other animals developed metastases, as observed 21 days after the onset of the experiment. Similar results were obtained in another experiment with the administration of homogenates of *Drosophila* embryos at the blastodermal stage to mice injected with Lewis lung tumoral cells: 15 days after the inoculation of cells, the primary masses were reduced in the treated animals, with a 35% decrease of the tumor weight.

The evidence that the pregnant uterus homogenate abrogated tumor cell growth, whereas the isolated mammal embryo homogenate did not induce any slowing effect, suggested that the factors involved belong to the mother-embryo cross-talk molecular network, and that breaking this interaction abrogates the anti-proliferative effect. Turning to an oviparous model, it was observed that the embryo alone delivers proliferation-slowing factors, although less efficient than those of pregnant uterine mucosa. It may well be that the uterus-embryo system of viviparous animals represents a further evolution of an intrinsic embryonic capability of keeping abnormally proliferating cell clones silent.

Conclusions

The *in vitro* and *in vivo* experimental data show that abnormal cell proliferation is somewhat affected by factors that are found in embryos and / or in pregnant uteri. These factors are organized in a network whose complexity should be unscattered to retain its full efficacy. This is particularly true for embryos, whose complex of

molecular factors represents a closed microenvironment that can normalize the behavior of abnormously growing cell populations via a regulatory process involving key-role proteins of cell cycle homeostasis. As for the molecules that characterize mother-embryo cross-talk, they may mediate a more rapid, apoptosis-enhancer process which does not necessarily need the integrity of the network. In fact, LPF represents a low molecular weight fraction of the whole sow pregnant uterine mucosa, and it does slow down the tumor cell proliferation rate alone as well as the whole raw homogenate.

A fundamental aspect of our findings is that only networks present in differentiative stages of embryo development can delay tumor growth, since networks present in multiplicative stages are ineffective or even modulate a slight proliferative effect on cell lines.

Our future research work will be to isolate the single components of these networks. The characterization of each single molecule involved in this kind of regulation will be important to better understand the most subtle mechanisms of action of this networks. An interesting goal will be to focus on the role of stem cells. A recent article shows that the proliferation of neural stem cells is strongly regulated by a homeobox transcription factor, Emx2, the mammalian homologue of *empty spiracles* of *Drosophila*, a fundamental gene for the correct embryonic development (8). This is further demonstration of the strict correlation between embryonic networks of differentiation and stem cells. Stem cells are embryonic cells that can be committed to different cellular types according to the network of factors that constitute the surrounding microenvironment. For example, neural stem cells can be differentiated into cells of the hemato-poietic lineage when put into contact with the hematopoietic microenvironment (9), or into skeletal muscle cells when put into contact with factors of skeletal muscle differentiation (10). Similar lines of evidence were observed with stem cells that are other than neural, e.g., liver stem cells (11). Thus, the knowledge of pathways and networks of stem cell differentiation may provide information about the processes of tumor cell differentiation, which is the basis for the normalization of cancer cells to a normal phenotype, by-passing mutations that originate malignancy.

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Post-Translational Modifications of the Retinoblastoma Protein (pRb) Induced by In Vitro Administration of Zebrafish Embryonic Extracts on Human Kidney Adenocarcinoma Cell Line

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The *in vitro* administration of embryonic extracts to tumor cells is known to induce a slowing down of the proliferation rate. In this work, the implications of this effect at a molecular level were considered, investigating the role of the Retinoblastoma protein (pRb). A kidney adenocarcinoma cell line, ACHN, was tested with extracts of zebrafish embryos taken during a differentiative stage of development. Whole cell lysates were analysed by immunodecoration with a specific α-pRb monoclonal antibody after SDS-PAGE and Western Blotting. Antibody staining showed that after 48 hours of treatment cells had a remarkably altered pRb phosphorylation profile, the hypo- and unphosphorylated forms being more dominant than hyperphosphorylated pRb. At functional level, unphosphorylated pRb keeps cells in a non-proliferative state by inactivating transcriptional factors of G_1-S transition, in line with the observed slowdown of the proliferation rate. This is evidence that embryonic factors act directly on important regulators of the cell cycle, reinforcing our view that the "physiological gene therapy" of cancer is promising.

Introduction

It is now well accepted that tumor cells share several key-role features with embryonic cells. These features are not only limited to the expression of specific surface markers, named oncofetal antigens (1), but also involve the activation of signalling pathways involved in cell multiplication and differentiation processes, including the APC/β-catenin/TCF/Wnt pathway (2,3) and the Hedgehog /Smoothened/Patched pathway (4). Moreover, a number of oncogenes and tumor suppressor genes play important roles in the normal development of tissues and organs (5,6).

The evidence that tumor development is reduced or even suppressed in embryos during early differentiation processes (7)

has led us to suggest that factors present in the developing embryo may affect tumour growth. Experiments carried out in our lab showed that treatment of several tumor cell lines with embryonic and/or pregnant uteri homogenates inhibit tumor growth both *in vitro* and *in vivo* (8,9,10), and that an important effector of cell cycle homeostasis, p53, is involved via alterations of its expression levels (11). We have suggested that a regulatory effect of embryonic factors on tumor cells may take place at molecular level, involving important regulators of cell proliferation and differentiation.

Among the proteins involved in the control of cell proliferation, the product of the Retinoblastoma (*RB*) gene, pRb, plays a pivotal role in regulating cell progression along the cell cycle phases (12,13). pRb is a nuclear protein which, in its active hypophosphorylated form, binds to and inhibits transcriptional factors of the E2F family. Since E2F factors mediate the G_1-S transition of cells, pRb is a negative regulator of cell growth because it retains cells in a non-proliferative state. For the same reason, pRb belongs to the tumor suppressor gene products. When a cell proliferation stimulus occurs, pRb is phosphorylated by specific cyclin/cyclin-dependent kinase (cdk) complexes, thus releasing from E2F factors which are eventually activated to successfully drive the cell through the G_1-S transition (14,15).

Since pRb may be one candidate target to the embryonic molecular regulation of tumor growth, the aim of this work was to investigate pRb behavior after the treatment of a human kidney adenocarcinoma cell line, ACHN, with homogenates of zebrafish embryos taken during one differentiative stage of development.

Materials and Methods

Cells and Media

A human kidney adenocarcinoma cell line, ACHN, was purchased from the Istituto Zooprofilattico Sperimentale of Brescia, Italy, and expanded in our lab at normal culture conditions (37 °C, 5 % CO_2). Culture media were composed of Eagle's Minimum Essential Medium (E-MEM), supplemented with 10 % Fetal Calf Serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids, and antibiotics. All media, sera and ancillary products were purchased from Labtek Eurobio.

Zebrafish Embryo Extracts

Zebrafish embryos were collected about 6 hours after egg deposition and fertilization, at the 50 % epiboly developmental stage, and dissolved with an Ultra-Turrax turboemulsionator in cold Phosphate Buffer Saline (PBS). The resulting homogenate, called "stage I Zf extract", was cleared from cellular debris by centrifugation, then brought to a 1 mg/ml total protein concentration and finally stored in aliquots at –20 °C until use.

Cell Proliferation Assay

Cells for the proliferation assay were seeded onto 60 mm petri dishes and let grow until they had reached about 60-80 % confluency (0 hours endpoint). Then they were treated with 1 µg stage I Zf extract and incubated for 24 and 48 hours. At these endpoints, cells were detached by a trypsin 0.025% solution and manually counted by the Trypan Blue dye exclusion method. Three petri dishes per treatment group were counted, and ten distinct counts per petri dish were performed. Cell count mean values were normalized referring to the 0 hours endpoint value as 1, and a cell proliferation / viability

percentage histogram was drawn assuming a 100 control value.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western Blotting, and α.pRb Immunostaining

Cells for pRb immunological detection were plated onto 100 mm petri dishes and treated with 3 µg of stage I Zf extract at 24 and 48 hours after reaching 60-80 % confluency. At the 0, 24 and 48 hours endpoints, cells were washed in cold PBS, collected by scraping, pelleted by centrifugation and finally lysed in cold detergent-based extraction buffer composed of 50 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5 % Igepal-CA630, and supplemented with a protease inhibitor cocktail and 0.5 mM PMSF immediately prior to use. Whole protein content of cell lysates was measured by Bio-Rad BCA method. Cell lysates were run in a 5% SDS-PAGE (loading: 30 µg proteins per lane) on a Bio-Rad Mini Cell Apparatus, according to the usual procedure of Laemmli (16). Finally, fractionated proteins were transferred onto a nitrocellulose filter by Western Blotting, according to the standard procedure described by Towbin *et al.* (17).

The filter was quenched in a blocking buffer composed of 5 % non-fat milk (NFM) in Tween XX-PBS (NFM-T-PBS), and then probed with a mouse α-pRb monoclonal antibody at a 1:1000 dilution in NFM-T-PBS for 4 hours. Alkaline Phosphatase (AP)-conjugated α-mouse secondary antibody incubation at a 1:2000 dilution in NFM-T-PBS for 1 hour was performed and a colorimetric detection step using a Nitro Blue Tetrazolium / 5-Bromo-4-Chloro-3-Indolyl Phosphate mix (NBT/BCIP) was carried out. NFM, primary and secondary antibodies were purchased from Santa Cruz Biotechnologies, NBT and BCIP were purchased from Roche Biochemicals.

Results

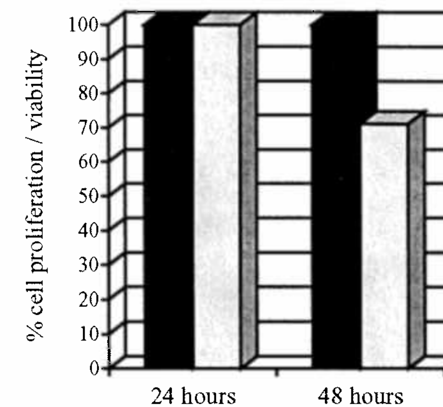

Figure 1: Histogram showing the cell proliferation / viability of ACHN percentage after 24 and 48 hours treatment with 3 µg of stage I Zf extract. Untreated control cells are black, treated cells are grey. Percentage values of control cells are assumed to be = 100. 24 hours after the treatment no differences are distinguishable between the values of untreated and treated cells. After 48 hours, treated cells show a percentage proliferation value of 71.1 %, corresponding to a 28.9 % inhibition value.

In order to quantify the effect of the administration of zebrafish embryo extracts on cell growth, a histogram showing cell proliferation/viability percentage was drawn after the treatment of ACHN cells with 3 µg of stage I Zf extract (figure 1). 24 hours after administering the extract, treated cells showed no decrease of the cell proliferation / viability index compared with untreated control cells. After 48 hours, treated cells showed a proliferation percentage value of 71.1, corresponding to a 28.9 % decrease in cell viability.

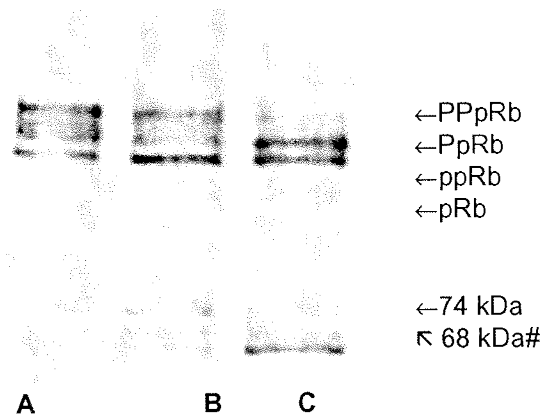

Figure 2: pRb immunostaining of ACHN whole control and treated cell lysates after fractionation by 5% SDS-PAGE and Western Blotting. 30 µg total proteins per lane were loaded. Lane A: untreated control cells. Lane B: 24-hour treated cells. Lane C: 48-hour treated cells. In lane A a group of almost four bands between 110 and 100 kDa is stained with equal intensity. A group of bands ranging from 74 and 68 kDa is very faintly stained. In lane B the lowest band of the 110-100 kDa group is more intensely stained, while intermediate bands show minor staining. Bands of the 74-68 kDa range are more visible. In lane C the two lowest bands of the 110-100 kDa group are strongly stained by the antibody, while the two upper bands are very faint. In the 74-68 kDa range, the lowest 68 kDa band is strongly stained, while the others are hardly visible. PPpRb: hyperphosphorylated pRb; PpRb and ppRb: intermediate hypophosphorylated forms of pRb; pRb: unphosphorylated pRb.

In order to investigate whether the effects of the administration of zebrafish embryo extracts on tumor cell proliferation involve pRb function, ACHN cells were treated with 3 µg of stage I Zf extract for 24 and 48 hours, then lysed, their whole proteins submitted to 5 % SDS-PAGE and Western Blotting, and finally immunodecorated with a α-pRb monoclonal antibody. As shown in figure 2, two groups of discrete bands were antibody-positive, one at the 68-74 kDa molecular weight (MW) range and one at the 100-110 kDa range. In control lane A, four bands in the 100-110 kDa range could be distinguished having equal staining intensity, while bands in the 68-74 kDa range were hardly visible. In the 24-hour treated cells, lane B, the 100-110 kDa banding pattern was retained, but the staining of the lower 100 kDa band was more intense. The bands in the 68-74 kDa group were more distinguishable than those in the control lane. In 48-hour treated cells, lane C, the 100-110 kDa range banding pattern was differently stained, the two lower bands being much more pronounced, while the two upper bands were hardly distinguishable. The 68 kDa band was much more intensely stained than the other bands of the 68-74 kDa range.

Discussion

The molecular and functional features linking embryonic and tumor cells have become more apparent in recent years. Not only do they co-express surface antigens, but they also share important regulatory proteins of cell multiplication and differentiation. On the basis of these findings, cancer can be viewed as a developmental disease and tumor cells can be considered as mutated undifferentiated embryonic-like cells, which have lost the ability of correctly couple proliferation and differentiation programs. In a previous work, we observed that the in vitro treatment of several human tumor cell lines with zebrafish embryonic extracts taken during distinct differentiation stages of development actually slowed their proliferation rates, whereas the treatment of the same cell lines with extracts taken during a merely multiplicative stage did not have any appreciable slowing effect (10). We hypothesized that the network of factors which are present in the developing embryo, could be able to "normalize" the cell cycle of poorly-differentiated tumor cells, leading them to a re-differentiated state, and that key-role effectors of cell cycle homeostasis could be the molecular targets of this event.

The proliferation rate of ACHN cells incubated with 1 µg of extract of zebrafish embryos collected at near 50 % epiboly, the developmental stage corresponding to the onset of gastrulation, is significantly affected only 48 hours after treatment, with a slowdown percentage value of about 30 %. At a microscopic observation, cells did not exhibit symptoms of suffering due to apoptotic or necrotic events (data not shown), suggesting that a softer, regulative phenomenon rather than a cytotoxic event has occurred.

The SDS-PAGE analysis of whole cell lysates suggested that the changes of the pRb electrophoretic banding pattern, as observed after immunodecoration with a specific monoclonal antibody, are due to post-translational affects at its phosphorylation state level. pRb exists in multiple phosphorylated forms, each reflecting a distinct functional role during the cell cycle. Since this experiment was carried out with an asynchronous cell population, the multiple-band pattern of pRb would represent the different phases of the cell cycle, with each band of the 100-110 kDa pattern related to a different phosphorylation state of pRb: the highest band being the hyperphosphorylated form, the lowest band being the unphosphorylated form, and the middle bands being intermediate phosphorylated forms. The evidence of the immunodecoration assay agrees with the results of the cell proliferation assay, in that only very subtle changes of the pRb phosphorylation pattern in the treated cells are distinguished after 24 hours compared with the control cells, as no growth inhibition is observed, whereas alterations of the relative staining distribution are visible 48 hours after the treatment, along with a 28.9 % decrease in the cell proliferation / viability value.

We argue that these alterations are not due to shifts of single electrophoretic mobilities. In fact, novel antibody-positive bands in the 100-110 kDa MW range did not appear. Thus, our interpretation accounts for a change of the relative ratio of differently phosphorylated forms of pRb rather than for the result of apoptosis-related proteolytic cleavage events. In 48-hour treated cells, hypo- and unphosphorylated species of pRb are more represented than the hyperphosphorylated form, indicating that cells with slower cycles are more than those actively proliferating. We also distinguished a group of bands with MW ranging from 68 to 74 kDa which were stained by α-pRb in the treated cells. Caspase-dependent proteolytic cleavage at the N-terminus of pRb originates a 5 kDa fragment and a 100 kDa truncated species, which is often hard to distinguish from the hypophosphorylated form (18,19). Furthermore, apoptotic

cleavage of pRb is accompanied by dephosphorylation, followed by the expression of a 68 kDa truncated form (20). Anyway, this 68 kDa species is also reported to be not necessarily derived from apoptosis (21). Actually, we cannot exclude a priori that a 5 kDa fragment is present in the whole electrophoretic pattern of treated cells, although it is not visible on a 5 % polyacrylamide gel, and also that apoptotic events have occurred; but it is clear from our results that a regulatory event, mediated by zebrafish embryonic factors, has affected ACHN cells via induction of pRb net dephosphorylation. At a molecular level, dephosphorylated pRb binds to and inhibits E2F transcriptional factors, blocking the cell cycle at the G_1-S transition phase; at a cell population level, this causes the net proliferation rate to slow down. Along with our previous work on p53 regulation by embryonic extracts (11), this is a preliminary attempt to elucidate the biochemical and molecular foundations of what we have already described as a "physiological gene therapy" of cancer (10). Our work still involves addressing many questions and goals, the most immediate being 1) to identify the single regulatory factors that are present in the embryonic microenvironment; 2) to elucidate the tumor cell molecular pathways affected and normalized by this regulation; 3) to investigate what regulatory molecules of the pRb homeostasis cascade system elicits this embryonic factors-induced dephosphorylation.

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Embryonic Differentiation Factors with Anticancer Properties: Preliminary Clinical Results in the Therapy for Advanced Tumors

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Experiments on animals have demonstrated that regulatory factors can stop or delay tumor growth. These regulators are present in the embryos of ovipara and in the pregnant uteri of mammals during differentiative processes and can regulate the anti-oncogene p53 and pRb in different tumor cells in vitro. These regulators are probably specific for each kind of tumor. A redundancy of these regulators was used in human therapy in an attempt to stop or delay the progression of the disease. The therapy consisted in the sublingual administration of low doses (from 9 micrograms/day to 12 micrograms/day of total proteins) of Zebrafish embryonic extracts at the stages of middle-blastula-gastrula, 5 somites, 20 somites in a glyceroalcoholic solution. The cases treated were seriously ill patients on whom oncologists had stopped traditional efficacious cancer therapies and patients with significant metastasis but still being treated with chemotherapy, radiotherapy or thermotherapy. Patients at the initial stages of disease were not included in this trial. In 3 years we treated 200 patients. Results: 1) 80% of the cases showed an improvement of the performance status, 2) the survival curves demonstrated a stabilization of the disease in a certain number of the final-stage cases 3) 8% of the cases demonstrated a regression of the tumoral masses. This clinical trial represents an open study and as such final conclusions cannot be drawn, except for the non-toxicity of the therapy. In fact, no adverse effects were observed in all the patients treated. A case-control study is now underway.

Introduction

It has been reported in literature (1,2,3,4,5,6) and demonstrated in vitro and in vivo (7,8,9, 10) that factors present during embryonic organogenesis can reduce or suppress tumor growth. This is probably because the substances taken from the embryo during cell differentiation can regulate important genes of the cell cycle. In fact, it was possible to activate the p53 onco-suppressor gene after treatment with embryonic extracts on different tumor cell lines in vitro (11). In addition to this

transcriptional regulation, a post-translational regulation of pRb on the human kidney adenocarcinoma cell line has been reported in another article in this issue. The evidence that embryonic factors of cell differentiation can be used as a" physiological gene therapy" of cancer constituted the objective basis to prepare a therapy to test in human cancer for compassionate use. As a result, different products containing specific embryonic differentiation factors, were prepared.

The aim of this paper is to illustrate the therapy and the preliminary results in humans.

Description of the therapy-Patients and methods

The products used for human therapy are Zebrafish embryo extracts in glyceroalcoholic solution.

Each solution has extracts of the embryos respectively at the stages of middle blastula-gastrula, 5 somites, 20 somites.

The Zebrafish embryos are washed in distilled water and placed in a solution of pure glycerin and 30 % ethylic alcohol. The proportion is 85 % and 15 % respectively.

The embryos are sonicated with 2 cycles of 10 seconds each and further treated with a turboemulsifier for 3 minutes.

These solutions are diluted with ethylic alcohol 30 % at the ratio 1 to 10: the final concentrations of total proteins must be about 3 micrograms/milliliter.

The therapy consists of the sublingual administration of 9-12 micrograms / day of total proteins obtained from the stages of middle-blastula-gastrula, 5 somites, 20 somites : the patients were usually given the substances from the middle-blastula-gastrula stage (66 %) and the substances from the other stages (34 %, ie17 % from the 5 somites stage and 17 % from the 20 somites stage).

The cases treated were :

1. seriously ill patients on whom oncologists had stopped traditional efficacious cancer therapies;
2. patients with significant metastasis but still under chemotherapy, radiotherapy or thermotherapy treatment.

Patients at the initial stages of disease and well controlled by traditional therapy were not included in this clinical trial.

In 3 years we treated 200 patients.

The following was recorded for each patient:

a) the performance status using the E.C.O.G. (Eastern Cooperative Oncology Group) system of evaluation.

b) the survival time from the beginning of the therapy. The Kaplan-Meier method was used to describe the survival curves.

c) The dimensions of masses of primary tumors and their metastases.

Results

The cases treated are shown in Table 1.

Tab. 1 - Cases treated with embryonic extracts.

60 breast cancer	8 pancreas cancer
23 lung cancer	6 myeloma
18 colon cancer	6 bladder cancer
15 hepatocarcinoma	4 ovary cancer
15 stomach cancer	4 leukemia (1 myeloid acute,
11 prostate cancer	3 myeloid chronic)
9 lymphomas (8 Hodgkin; 1 n.H.)	1 larynx cancer
9 glioblastoma	1 melanoma
9 kidney cancer	1osteosarcoma

60 of these cases were under thermotherapy treatment. 19 cases were under chemo-radiotherapy treatment.

80 % of the treated patients improved their performance status (generally from the 4 or 3 status of E.C.O.G. scale to the 2 or 1 status). In addition, the patients reported a reduction of pain and a decreased intake of analgesic drugs.

The masses of primary tumors or of their metastases decreased in the following cases: breast cancer (4 cases), lymphoma non Hodgkin (1 case), lymphoma of Hodgkin (1 case), cancer of the colon (2 cases), stomach (1 case), lung (1 case), kidney (1 case), bladder (2 cases), prostate (1 case under goserelin therapy), larynx (1 case), osteosarcoma (1 case).

The survival curves are reported only for the most numerous subgroups of cancers in the whole group.

Discussion

This clinical trial is an open study that is usually made before a case -control study. Final conclusions cannot be drawn without the control group, except for the non-toxicity of the therapy. In fact, no adverse effects were observed in all the patients treated.

Other observations can be made at the end of the three-year period. It can also be stated that the therapy has some positive effects on performance status. In fact, 80 % of the patients improved their performance status and reported less pain.

These consequences cannot be ascribed to the placebo effect only, because their incidence is higher than the placebo effect.

Conclusions about the reduction of tumoral masses are more difficult to make. In fact, the patients treated were at a very advanced stage of the disease. 60 of these patients were under thermotherapy and 19 under chemo-radiotherapy, the other 121 patients had just finished chemotherapy or radiotherapy. The effects on the dimensions of tumoral masses could be ascribed to chemotherapy or radiotherapy. However, a very advanced kidney adenocarcinoma, with significant pulmonary, bone and surrenalic metastases demonstrated a complete regression of tumoral masses, without chemo-radiotherapy. The complete remission of the disease is still present after four years from the beginning of the therapy.

The survival curves demonstrated a high percentage survival rate after several months for each kind of tumor. These percentage survival rates are high, if we consider that the diseases were very advanced and that the life expectancy in these cases was generally no more than 6 months. Therefore this therapy seems to have a positive effect on the survival of the patients.

However, in order to clarify these aspects of the problem we are now conducting a case-control study.

This therapy can be considered complementary to the other efficacious cancer therapies.

We are also studying how to improve its efficiency: each kind of tumor probably requires a specific regulation network.

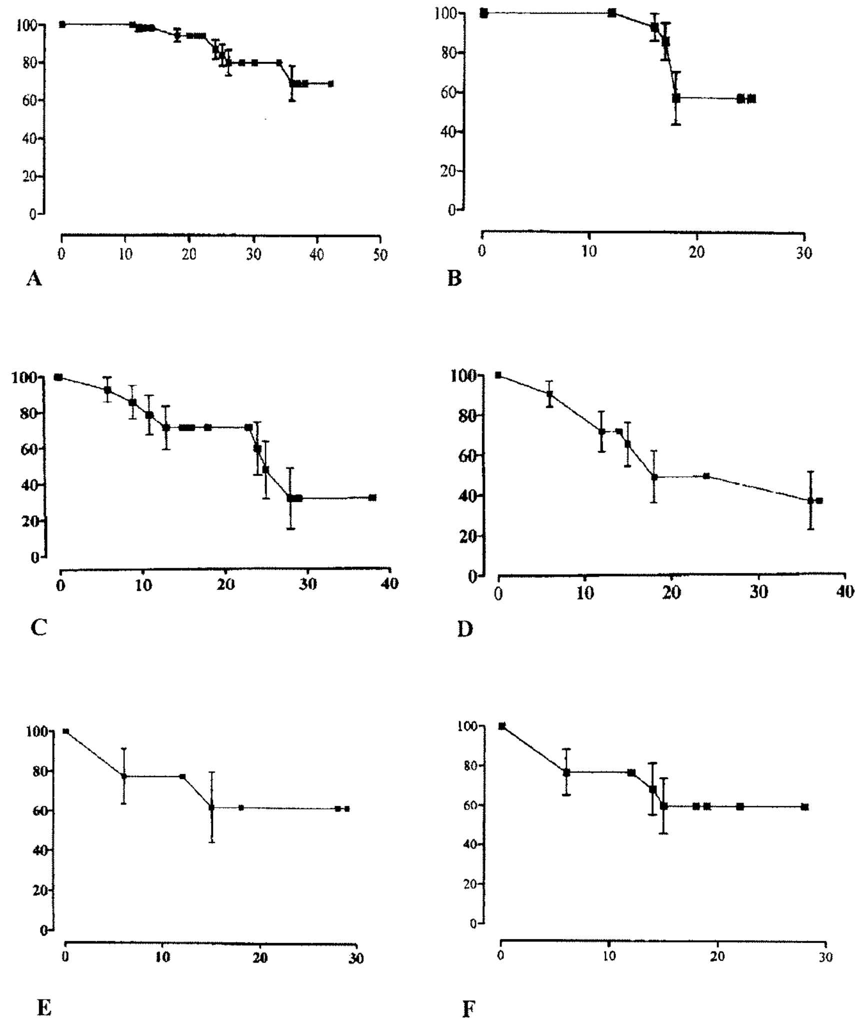

Figure: Percentage survival curves of patients with A) breast carcinoma; b) colon carcinoma; C) hepatocarcinoma; D) lung carcinoma, E) prostate adenocarcinoma; F) stomach carcinoma. Abscissa axis: months of treatment. Ordinate axis: percentage survival rate.

Fig. 1A shows the survival curve for 60 patients with breast cancer. After 40 months from the beginning of the treatment the percentage survival rate is about 80 %.

Fig. 1B shows the survival curve for colon cancer. The percentage survival rate is about 60 % after 24 months of treatment.

Fig. 1C shows the survival curve for hepatocarcinoma. The percentage survival rate is about 35 % after 40 months of treatment.

Fig.1D shows the survival curve for lung cancer. The percentage survival rate is about 40 % after 36 months of treatment.

Fig. 1E shows the survival curve for prostate cancer. The percentage survival rate is about 60 % after 30 months of treatment.

Fig. 1F shows the survival curve for stomach cancer. The percentage survival rate is about 60 % after 30 months of treatment.

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The Embryonic and Maternal Regulatory Factor as A Palliative Therapy for Advanced Solid Tumors

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Previous research, performed on animals as well humans, has suggested that embryonic regulatory factors are effective in inhibiting tumor growth and/or in improving performance status or survival in advanced cancer patients. A set of different regulatory proteins were extracted from fish embryo during differentiative processes (middle blastula-gastrula, 4 somites, 20 somites), sharing a well-expressed ability to reduce tumor growth and to enhance the expression of the anti-oncogene p-53 in several different tumor cell lines. Forty-seven advanced cancer patients, refractory to conventional cures, entered the study and were treated with a product containing a solution of extracts of zebrafish embryos and of pig decidua, antioxidants and vitamins A, B1, B6. The product was administrated by sublingual absorption of 1 ml / three times a day. After three weeks of treatment a significant improvement in performance status was observed in 80% of patients. The benefit was almost unchanged after 6 months of treatment and general conditions worsened only in 30% of the cases. After the first three months of therapy partial objective responses were recorded, with a significant reduction in the size of tumor-mass, in 4 patients. Tumor size in 70% of patients did not change or showed only a minimal reduction, while progression was observed in 20% of patients. The overall survival rate was 80% after 8 months of therapy. The side effects were negligible. No definitive conclusions can be drawn from this preliminary report even if the results are promising and further studies are needed to demonstrate the significance of the results.

Introduction

A neoplastic cell is characterized by abnormal cell proliferation and the impairment of several differentiating processes. Most tumors share a marked heterogeneity in structural morphology, biochemical phenotype and cell functions, even if a certain, albeit abnormally small number of the cells has the expressing characteristics of differentiated cells. Many studies have demonstrated that cancer cells can be induced to perform differentiating pathways, recovering biochemical and functional characteristics of the normal phenotype, including the ability to express apoptotic mechanisms (1). Several agents (retinoids, polar-apolar compounds, vitamin D_3, melatonin, growth factors, TGFβ-1, etc.) can induce differentiation or inhibit the proliferation of transformed neoplastic cells (2,3,4). Noteworthy is the fact that terato-

carcinoma, leukemia or adrenal medulla carcinoma cells differentiate to normal tissues when placed in a normal embryonic environment (5,6,7). The rationale of a use for differentiating factors in the treatment of human cancers is largely based on laboratory studies that have demonstrated the effectiveness of these substances in inducing a wide variety of tumor cells to differentiate and/or to stop growing, even if the clinical response to these agents is likely to involve more complex effects than those observed in vitro (8). We are aware of the fact that differentiating mechanisms are controlled by a network of several "informational" molecules that cooperate in order to restore a correct relationship between cells and tissues, and to ensure a physiological "biochemical dialog" that involves the complexity of the organism as a whole.

Recently it has been demonstrated that tumor development induced by a wide class of carcinogenic compounds is reduced or suppressed when the differentiating processes are in progress (9,10); once organogenesis is complete, the frequency of tumor induction rises constantly accompanied by a decrease in the rate of malformation (11). Some protein factors expressed by the embryos of ovipara (12) or in the pregnant uteri of mammals (13) shared a marked inhibitory action on the proliferation of cancer cells, activating the p-53 anti-oncogene in different tumor cell lines in vitro (14). A previous clinical trial (15), performed on advanced cancer patients refractory to chemotherapy or radiotherapy and treated palliatively with zebrafish embryo extract, demonstrated some important benefits in terms of performance status and overall survival. The present open clinical trial was pointed out to confirm this preliminary data in human tumors.

Patients and Methods

Protein glyceroalcoholic extracts are obtained from zebrafish embryos collected during organogenesis and from pig decidua at 23 days of pregnancy. Solutions were prepared as previously described (15). Vitamins (retinoic acid, pyridoxine, thiamin) as well as magnesium, α-lipoic acid, aminoacids (cysteine and methionine) were added to obtain the final pharmacological solution according to Biava and colleagues. The treatment was a 1 ml solution (3 μg of embryo proteins and 150 μg of pig decidua proteins) three times a day, administered sublingually, to fasting subjects. Furthermore, each patient received personalized supportive care in order to control pain, depressive symptoms or to alleviate side effects related to the progression of the disease.

Forty-seven advanced cancer patients entered into the study and 45 met the criteria for statistical evaluation; 5 out 45, affected by neoplastic diseases of the central nervous system (glioblastoma multiforme), were excluded considering the fact that, after three months of therapy, they subsequently underwent surgery a second time; in these cases the histological character and malignant grade of the tumor were re-evaluated. All patients are in progression after several trials (more than 2) of chemotherapy or radiation therapy, and have discontinued traditional cures. The follow-up, ranging from 3 to 9 months, averages 6 months. For each subject, the performance status (using ECOG criteria), objective response rate (using NCI criteria, by means of Tc scan and tumor markers estimation), survival time and life expectancy curves (using the Kaplan-Meier method), were recorded.

Results

Performance status

Forty patients met the eligible criteria for statistical evaluation. A significant and dramatic improvement in the performance status was observed in the majority of cases (80%) after the first three weeks of therapy. The benefit was almost unchanged after 6 months of treatment and general conditions worsened only in 10% of the cases (30% of the patients after 6 months). Weight loss stopped in 22/36 patients who previously had lost at least 20% of their weight; anorexia was reduced or disappeared in 18/30 patients; asthenia was reversed in 30/41 patients. A general improvement in sleeping parameters as well as in depressive symptoms was observed in 15/29 subjects.

Objective Response Rate

After the first three months of therapy, partial objective responses were recorded, with a significant reduction in the size of tumor-mass (>50% of the initial values), in 4 patients affected by advanced colon cancer with peritoneal metastasis (Fig.1). In two cases, in the following 3 months, a further reduction was obtained and the patients were surgically treated in order to remove the residual pelvic mass; it could be pointed out that the only objective response was obtained promptly, during the first months of therapy and in no case was observed after the fourth month; nevertheless, no progression was observed in the group of responding patients after 8 months of therapy. Tumor size in 70% of the patients did not change or showed only minimal reduction (<20% of the initial mass); the stabilization of the disease was maintained in this group for at least 6 months; the incidence of progression reached 20% after three months of therapy and then, after 6 months, rose to 30%.

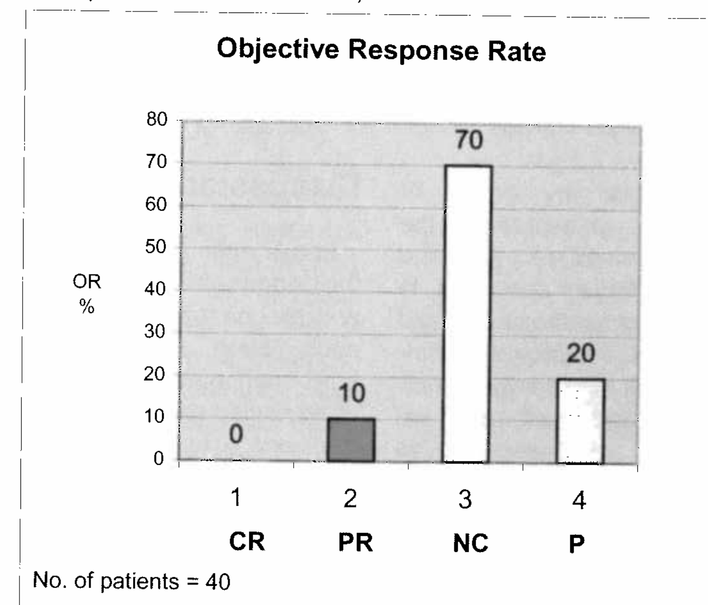

Figure1: Objective Response Rate in advanced cancer patients receiving zebrafish embryonic extracts. The response was evaluated after three months of treatment using NCI criteria. Legend: OR = objective response; CR = complete response; PR = partial response; NC = no change; P = progression.

Survival

Overall survival data (Fig. 2) demonstrated that after 8 months of treatment 80% of patients were still alive. Life expectancy curves showed a small and progressive decline in the survival rate, but with significant differences compared with historical control (data not shown) for which survival incidence slowed down dramatically during the first three months after the interruption of traditional treatment (50% of patients alive at 3 months and only 12% after 6 months).

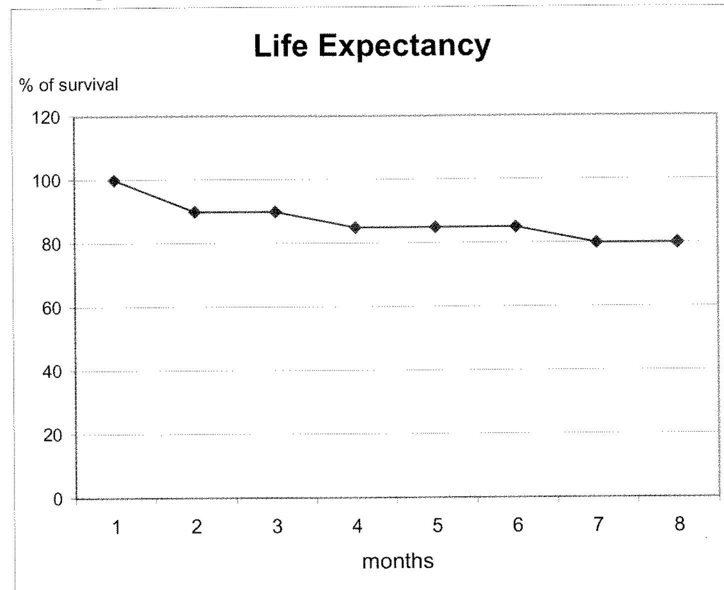

Figure 2: Life Expectancy in advanced cancer patients receiving the zebrafish embryo extract. Evaluable patients after 8 months of therapy n = 40.

Histological modifications

Five patients affected by grade IV multiform glioblastoma, widespread in the cortex and in progression after surgery and radiotherapy were treated as described, in association with desamethazone and mannitol. Neurological symptoms (headache, sensibility as well motor impairment) improved significantly and in all cases we decided to perform novel surgery to remove the residual mass. The histological results differ significantly from those obtained during the first surgical removal: in all cases the histological grading moved from IV to II. All the patients continued the medical treatment and are still alive, without any sign of progression, 4 months after the second surgical operation.

Discussion

In this open study we demonstrated that the administration of embryo zebrafish and pregnant pig decidua protein extracts could exert some positive effects on performance status and the overall survival of advanced cancer patients not eligible for traditional cures. Performance status improved quickly and dramatically in a percentage of patients higher than that expected when a placebo effects occurs. In fact this benefit persists even after 6 months and slows down slowly only after this period. Overall survival is better than that generally expected for this category of patients: historical control, in fact, shows that after 6 months, only a minimal amount of patients are still alive (12%), meanwhile, with the suggested treatment, the overall survival rate is 80%. It must be underlined that in no case did the therapy have to be discontinued and no relevant side effects were recorded, with the exception of rare cases of subjective taste aversion and, in 4 cases, mild and transient abdominal pain.

Advanced cancer patients suffering from disseminated disease, mild or important multiple organ impairment and - last but not least - from major side effects after multiple pharmacological, surgical or radiation treatments, are not candidates for further traditional cures and discontinue any form of therapy. Life expectancy in these cases is generally no more than 3-6 months. Keeping in mind this data, our study demonstrated not only a higher survival in treated cases, but also a little, yet significant, incidence of objective response (10%) with tumor-mass reduction higher than 50%. These results concur with the preliminary report presented by Biava (15) and co-workers and prompted us to start a well-designed randomized case-control study. It could be argued that a set of several different combinations of embryonic extracts should be prepared in order to improve the efficacy of the therapeutic protocol, bearing in mind that each kind of tumor probably requires a specific network of regulation to obtain the maximum differentiative and inhibitory effect. It is almost intriguing that the treatment could exert a well-defined modulatory action on differentiation processes, as demonstrated in the 5 patients affected by glioblastoma, in which, after 3-4 months of therapy, the histological examination performed on the tumor mass removed during surgery, revealed in all cases a significant change in the tumor grade. It could be argued that embryo extracts act as biological response modifiers, exerting some effects on genomic regulatory mechanisms, thus enhancing the recovery of a more differentiate phenotype and inhibiting the malignant behavior of the transformed cell. This data outlined that the transformation of cells does not necessarily destroy the potential for expression of differentiated characteristics including cessation of proliferation.

Our clinical trial represents an open study, based on a historical control group of advanced cancer patients who received supportive care only. A controlled randomized case-control study is needed in order to ascertain the statistical effectiveness of this biological approach in the treatment of human cancer.

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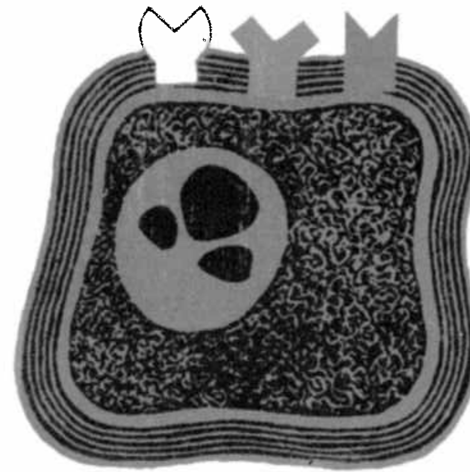

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