# Chalones: Chemical Regulation of Cell Division

Most normal cells either divide very slowly or do not divide at all. Cancer cells, in contrast, divide and proliferate very rapidly, eventually overwhelming normal cells by force of numbers. Chemotherapists have tried, with only partial success, to take advantage of this difference by using nonspecific antimitotic agents to destroy dividing cells, but there has been relatively little productive investigation of the molecular control mechanism that normally holds cell growth in check.

One simple, perhaps even simplistic, rationale for this phenomenon is the existence of chalones, endogenous mitotic inhibitors that control cell proliferation by negative feedback inhibition. That is, mature cells might synthesize and release a chemical messenger called a chalone; high levels of chalone within the tissue system would then inhibit further replication of immature cells. This general theoretical model was first proposed around 1957 by Paul Weiss of Rockefeller University, and has subsequently been refined and at least partially verified by a number of investigators, most notably William S. Bullough of London's Birkbeck College and O. H. Iverson of the Institute of General and Experimental Pathology, Oslo, Norway.

Early this month, they and some 50 other investigators—virtually everyone in the world who has worked on chalones—gathered under the auspices of the National Cancer Institute and the Office of Naval Research to compare their results and to debate the validity of the chalone concept. Their presentations (which will be published as a supplement to the *Journal of the National Cancer Institute*) suggest not only that the concept *is* very likely valid, but also that it offers tremendous, albeit far distant, potential for the control of a wide variety of diseases involving excessive cell proliferation.

The assembled group was unable to arrive at a rigorous definition of chalones (a term derived by Bullough from a Greek word meaning "to slack off the main sheet of a sloop to slow the vessel down"), but there is general agreement on the four principal characteristics of these substances.

► Chalones inhibit mitosis both in vitro and in vivo.

► Their action is reversible; they are not cytotoxic.

► They are synthesized by mature cells of the tissue upon which they act. Chalones are, however, released from cells and circulate in the blood stream and probably in humoral fluids. Bullough has, for example, isolated active epidermal chalone from both blood and urine.

► They are tissue-specific, but not species-specific. Bullough, Iverson, and Edna B. Laurence have found apparently identical epidermal chalones in extracts from a wide variety of mammals and even from fish. It is this property, if confirmed, that endows chalones with such great potential for chemotherapy—the ability to halt proliferation of one cell type without affecting others.

Chalone activity was first demonstrated in 1960 by Bullough and Laurence. They were searching for a hypothetical endogenous "hormone" that promotes cell division in the healing of epidermal wounds, but found instead an antimitotic substance, the removal of which stimulates healing. Their results were complicated by the fact that the epidermal chalone requires a hormonal "cofactor" for inhibition; subsequently discovered systems, however, do not show this requirement.

Bullough and Laurence's findings have been verified and extended in several laboratories, and the epidermal chalone is now undoubtedly the most thoroughly investigated of these compounds. Recent evidence, Laurence says, suggests that the chalone system is somewhat more complex than originally anticipated, and that the hormones are necessary to block a chalone neutralizing factor in the system.

Similar mitotic inhibitions have so far been observed in 15 diverse tissue systems, including lymphocytes, erythrocytes, fibroblasts, kidney, lung, liver, uterus, hair follicles, and sebaceous glands—in short, Bullough says, in every system where they have been sought. Tissue extracts from each of these systems slow proliferation of cultured cells from only that system, and in many cases a similar inhibition has been demonstrated in the corresponding tissues in animals. Iverson, for instance, found that complete removal of epidermis from one side of the wing of the African fruit bat strongly stimulates mitosis in epidermis on the opposite side of the wing, but that this increased mitotic activity is halted by sprinkling crude epidermal chalone on the wound. Anthony Chung of Georgetown University School of Medicine, Washington, D.C., has retarded the growth of the thymus in fetal rabbits (without affecting other organs) by injecting the animals with crude extracts from bovine thymus. And Bullough and Laurence have shown that wound healing in rats can be slowed or stopped with epidermal chalone.

The mode of action of chalones in such systems is largely conjectural, however. There is general agreement that chalone activity somehow depends on the state of cellular membranes, but the nature of this link is unclear. Many investigators, such as Bullough and Iverson, hold that the cell membrane serves to concentrate chalone within the cell. Others, such as John C. Houck of the Children's Hospital of the District of Columbia, argue that chalones are bound to the membrane, probably outside the cell. Both groups agree, however, that the interaction between membrane and chalone is the probable source of the tissue specificity of chalones. Both also agree that damage to the membrane, whether from wounding or from the action of a virus or carcinogen, causes the cell to lose its chalone, thereby stimulating growth that leads either to repair of the wound or to formation of a tumor.

This speculation is supported by several lines of experimental evidence. Dose-response curves for the inhibitors indicate that there is a specific receptor site for chalones within or on the cell. Cancerous cells, moreover, retain their ability to synthesize chalone—although possibly in reduced quantities—but most of this chalone is released into the blood stream, producing the apparent paradox that the concentration of circulating chalone is higher in diseased animals. Most important, such cells retain their ability to respond to changes in chalone concentration.

The most impressive demonstration of this response was presented by Tapio Rytomaa of the University of Helsinki, who examined a chalone from granulocytes of normal and chloroleukemic rats. (Granulocytes are a type of white blood cell produced by mitosis of progenitor stem cells in bone marrow; the granulocytic chalone inhibits this mitosis. Chloroleukemia is a carcinogen-induced form

of leukemia characterized by excessive production of granulocytes, and is maintained in the laboratory by subcutaneous transplantation of malignant cells in rats.) Rytomaa found that extracts from chloroleukemic granulocytes contain less than 10 percent of the amount of chalone in extracts from normal granulocytes, but that blood from the diseased animals contains much greater concentrations of chalone than does blood from normal rats.

Furthermore, he demonstrated that crude extracts of granulocytic chalone from both types of rat suppress granulocyte proliferation both in vitro and in vivo. Chloroleukemia is generally fatal within 12 days after transplantation, and spontaneous remissions do not occur. Of a dozen rats treated with crude chalone extracts for periods of as long as 2 weeks, however, three are still alive after 3 years, and the remainder had their lifespans extended significantly. The chalone itself cannot destroy a tumor, Rytomaa emphasizes, but by slowing or halting tumor growth it allows the host's natural defenses to reject the tumor. Such a mechanism is similar to that of the sulfonamide drugs, which merely stop bacterial growth so that the host can destroy the infection.

Similar but less dramatic regressions have been observed with a variety of other malignancies—including epidermal carcinomas and melanomas, other granulocytic leukemias, and lymphocytic leukemias—in each case by use of relatively crude extracts from the appropriate tissues. In each instance, moreover, similar extracts from other tissues had no effect, strong evidence that the tumor rejection was not an immunological response stimulated by impurities in the tissue extract.

The presence of impurities in the chalone prèparations is, nonetheless, the major criticism of virtually all chalone experimentation. In most instances, chalone is obtained simply by disrupting the appropriate cells and extracting the cell mass with water. Insoluble cellular material is removed by centrifugation, and more impurities are precipitated by the addition of ethanol. Lyophilization of the supernatant then leaves a fine powder containing the chalone activity, and most of the experimental work has been performed with this material. Further tentative steps toward purification have in most cases been unsuccessful.

The chalones thus isolated fall into two distinct classes. Many appear to be glycoproteins with a mass of about 30,000 to 50,000 daltons and an isoelectric point between *p*H 5.2 and 6.0. They are stable at low *p*H but are degraded at high *p*H or in the presence of proteolytic enzymes. and they are thermally labile. None of these compounds have been further purified, however, and some of the observed properties may be artifactual.

A smaller group of chalones have a mass of only 1000 to 3000 daltons. The two most studied members of this class are a granulocytic chalone, isolated from bone marrow cultures and bovine spleens by W. R. Paukovits of the University of Vienna, and a liver chalone isolated from rats by Walter G. Verly of the University of Montreal. They are currently the purest chalones available.

The granulocytic chalone isolated by Paukovits appears to be the same material investigated by Rytomaa. It is a heat-stable, hydrophilic polypeptide containing 20 to 30 amino acid residues. Preliminary results, Paukovits says, indicate that it has no sulfur-containing amino acids but that the amino acid distribution is otherwise normal. Verly's chalone shows similar physical properties. Both men hope to determine amino acid sequences of the chalones in the near future.

The discovery of two types of chalones has inevitably led to much speculation about the relation between them. Some investigators suggest that the size difference may simply reflect varying degrees of aggregation of small units. Others contend that the larger compounds are the complete chalones and that the glycoside residues provide a macromolecular structure that endows the biologically active polypeptide with tissue specificity. Another alternative is offered by Kjell Elgjo of the Institute of General and Experimental Pathology, who suggests that at least some tissue systems may have two chalones that act at different phases of the mitotic cycle. Peter Bichel of the Cancer Research Institute, Aarhus, Denmark, for example, has isolated chalones of both high and low molecular weights from Ehrlich ascites tumors and shown that they inhibit proliferation at different points in the mitotic cycle. Friedrich Marks of the Institut für Biochemie, Deutsches Krebsforschungzentrum, Heidelberg, Germany, has obtained similar results with pig epidermis, and D. L. Dewey of Mount Vernon Hospital, Middlesex, England, has isolated a low molecular weight melanocyte chalone that appears to inhibit at a different point in the mitotic cycle than does a previously isolated larger melanocyte chalone.

Much of the ambiguity surrounding the nature of chalones will be removed when pure, fully characterized materials are available, but the attainment of this goal is hindered by two major problems: the small quantities of chalone present in tissue, and the lack of a rapid, sensitive assay for chalone activity. The former problem is, of course, common in the isolation of biological macromolecules, and should become less important as more efficient separation methodology is developed. The latter problem is more difficult.

Measurement of cellular mitotic rates has traditionally been accomplished by one of two techniques. Early workers, particularly those investigating epidermal systems, used colcemid to trap dividing cells in metaphase, then visually counted the number of mitotic cells per unit area. This count provides an accurate measure of mitotic activity, but it is very tedious and time-consuming and is useful only in systems where an intact cell structure exists.

Most investigators now assay mitotic rates by measuring the incorporation of tritiated thymidine into cellular DNA on the commonly accepted assumption that DNA production rates parallel mitotic rates. This method is subject not only to the cost and technical difficulties generally associated with the use of radioisotopes, but also to inaccuracies arising from impurities in the chalone preparation. Contamination of the tissue extract by small nucleotides or thymidine-catabolic enzymes (such as thymidine phosphorylase), for example, leads to spuriously low values of [$^3$H]thymidine incorporation.

Both techniques, moreover, are subject to more generalized errors. Cytotoxic materials, for instance, will also inhibit mitosis, and it must be demonstrated that any observed inhibition is reversible. Tissue extracts injected into tumor-bearing animals may also be contaminated by bacteria or small nucleotides that could trigger a normal immunological rejection process which might be mistaken for a chalone reaction.

These problems are of such magnitude, in fact, that the National Cancer Institute recently awarded two grants and a contract for the development of isolation protocols for chalones and simple, efficient assay systems for chalone activity. Only when these techniques are developed and pure chalones are available for study, says an NCI official, will it be possible to assess the true significance of chalones.

—THOMAS H. MAUGH II