

## The Biochemistry of Cancer

Additional readings; pp. 682 (oncogenes); pp. 680 (Ras) ; and 916 (P53 and apoptosis)

### Introduction:

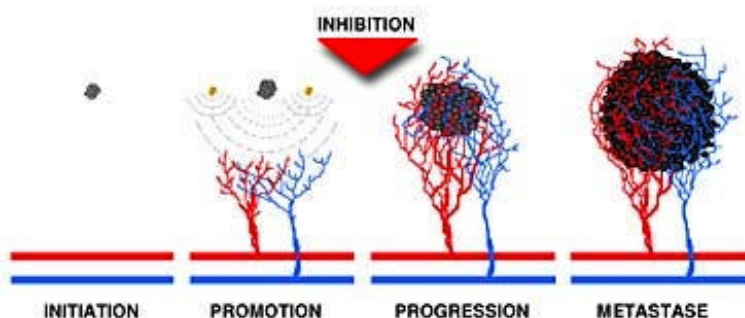
Cancer cells are characterized by three distinguishing properties: 1) a diminished or unrestrained control of growth; 2) invasion of local tissues and 3) the spread or metastasis of cancerous cells. Cancer is the second leading cause of death in the United States, with heart disease being the first. In fact, cancer accounts for approximately 20% of all deaths and thus is of considerable interest as an area of research. Biochemists study various aspects of the molecular events that are associated with cancer. Scientists in many other disciplines (e.g., chemistry, genetics, virology, and microbiology) also conduct research into the causes of and cures for cancer.

Tumors arise with great frequency, especially in older animals and humans, but most pose little risk to their host because they are localized and of small size. We call such tumors benign; an example is warts. It is usually apparent when a tumor is benign because it contains cells that closely resemble, and may function like, normal cells. The surface interaction molecules that hold tissues together keep benign tumor cells, like their normal counterparts, localized to appropriate tissues. Benign liver tumors stay in the liver. The cells are usually encapsulated and will delineate the extent of a benign tumor. Benign tumors become serious medical problems only if their sheer bulk interferes with normal functions or if

Cancer is a group of diseases in which cells grow in an uncontrolled way. It is described as a group of diseases

because cancer can originate in many different tissues in the body, and the characteristics of the disease may differ at different sites of origin. This fact alone has hampered the ability of scientists to understand cancer.

In describing cancer as diseases characterized by uncontrolled growth of cells, it is important to consider normal human development. From initial cells formed following conception, we develop into complex biological organisms with characteristic morphology, anatomy, physiology, and biochemistry. Thus, normal development, which includes differentiation of cells, must be under the control of some complex regulatory mechanisms. These mechanisms require a great deal of communication among various types of cells. After development, cellular communication also is important to prevent unregulated growth of cells. Cancer develops when these regulatory controls are lost.



they secrete excess amounts of bioactive molecules such as growth factors or hormones.

In contrast the cells which make up a malignant tumor express

proteins characteristic of the cell type from which it arose and a high fraction of the cells grow and divide more rapidly than normal. Some malignant tumors remain localized and encapsulated for at least a time. These tumors are not invasive and have not yet metastasized. An example is the carcinoma in situ. Most however do not remain in their original site; Instead they invade surrounding tissues, get into the body's circulatory system and set up areas of proliferation away from the original site. The spread of tumors and establishment of secondary areas of growth is called metastasis; most malignant cells eventually acquire the ability to metastasize. Thus the major characteristics that differentiate metastatic or malignant tumors from benign tumors are their invasiveness and spread.

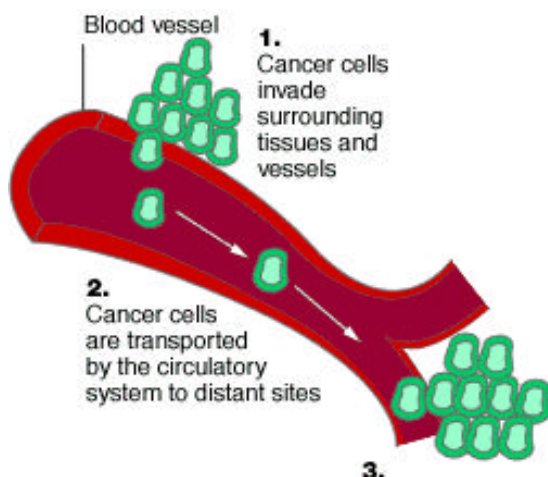
Cancer cells can be distinguished from normal cells by microscopic examination. They are usually less well differentiated than normal cells. They may have some but usually not all of the

proteins expressed in a normal cell. Some of the normal gene expression is lost during transformation (the act of a normal cell becoming a tumor cell or one that will live outside of its normal conditions). Malignant tumors are classified as carcinoma if they come from the skin, gut, nervous or respiratory tract cells. They are called sarcomas if they come from connective tissue, muscle or blood cells. Leukemias (white blood cell cancer), a class of sarcomas, grow as individual cells in the blood, whereas most other tumors arise from solid masses.

The restriction of a normal cell type to a given organ or tissue is maintained by cell to cell contact. Metastatic cells break these contacts with other cells in their tissue of origin and overcome the constraints on cell movement provided by the basement membranes. As a result, metastatic cells can invade adjoining tissues before spreading to distant sites through the circulation. These cells have lost their contact inhibition. That means that most cells, while needing to be anchored to a solid mass, will grow until there is a connection to the surrounding cells. Metastatic cells have lost this requirement and can therefore move throughout the body and spread.

### Causes of Cancer

Many factors may contribute to the development of cancer. Certainly genetics plays a role. For example, in some cases of cancer, there is a very strong familial association in that many individuals in a family may develop cancer. This observation suggests that the genetic make-up of an individual is related to the development of cancer. However, this familial association is not



observed in many cases of cancer. It is estimated that 30-90% of cancers are a result of environmental factors, though this broad range indicates that the role of environmental factors in development of cancer is rather controversial.

One line of evidence supporting a role for environmental causes of cancer comes from epidemiology, which is the study of the distribution of disease. Epidemiologists have studied the incidence of cancer in immigrants. Cancers of the breast, colon, and prostate are low in Eastern and African countries but are high in North America. In the United States, the incidence of breast cancer is 7 times that in Japan. However, immigrants develop the same pattern of cancer incidence as those in the country to which they emigrate, normally within 1 to 2 generations. From this evidence, one can argue that factors in the environment are very important in the development of cancer.

### Environmental Causes of Cancer

Carcinogens are substances and agents that cause cancer. As we consider chemicals as carcinogens, we also want to take a broad view of our exposure to chemicals. Certainly we may be exposed to carcinogens as a result of industrial activities. However, we also are exposed as a result of natural processes such as forest fires. Moreover, the food we eat may contain naturally occurring carcinogens as well as those present as food additives or that result from cooking.

### Evaluation of Carcinogenicity

How do we know that a chemical is a carcinogen in humans? This question is very difficult to answer definitively. One line of evidence comes from epidemiological studies as cited above. However, because humans are exposed to many different chemicals, it is often difficult to be certain that a given one is the sole cause of cancer.

To try to relate the carcinogenicity of chemicals to cancer, tests are carried out in experimental animals. However, a number of difficulties arise

in trying to extrapolate these studies to humans. First, high doses are administered to animals so that a significant number of tumors can be observed in a reasonable period of time. Second, animals may respond differently to particular chemicals. That is, some species of animals are more susceptible to development of cancer following exposure to certain carcinogens than other species. Thus, we cannot be certain how to extrapolate from animal studies to humans.

Additionally, because testing of animals are slow, expensive and obviously problematic, other methods of screening carcinogenicity of chemicals have been developed. One method is based on detecting the ability of a chemical to cause mutants in cell culture. The Ames assay used a specially constructed strain of salmonella that has a mutant gene that is necessary for the production of histadine. These particular salmonellae can therefore not synthesize histadine and the amino acid must be added to the media for the cells to continue to grow. Under these conditions (with additional histadine added) cells are exposed or treated with various potential carcinogens. When a mutation that occurs at His- gene, the latter mutation can restore its reading sequence converting it to the wild type that can survive and grow without histadine. Thus the number of mutations can be deduced by the number of growing colonies and an understanding of the genomic size of the organism. The advantage of this test is that it can be done very quickly (a few days) and it is very inexpensive to do.

### Oncogenes

Oncology is the study of cancer; hence genes associated with cancer are called oncogenes. All known oncogenes code for products that are associated in some way with cell growth and development. Recall our earlier discussion about normal development and the tight regulation that is required for development to proceed normally. It makes sense then that mutations in genes that code for products involved in development are likely to lead to changes that would result in cancer.

Another class of genes that are associated with development of cancer are tumor-suppressor genes. It has been recognized in the last few years that certain genes code for products whose role is to control cell division. If these genes (tumor-suppressor genes) are mutated, the altered gene product may not function normally and the cell will divide uncontrollably.

### Cancerous cells are almost always genetically altered

For cancers to develop one thing is certainly clear, we know that the cells of a tumor descend from a common ancestral cell that at some point, usually several years before the tumor developed became mutant. This program of multiple mutations, accumulate throughout the life of the host and each mutation is passed on to the daughter cells. For cancer to rise, a series of critical mutations must occur, usually through mutations in specific classes (proto-oncogenes and tumor suppressors). These mutations can take place by several different mechanisms including gene translocation, viral infection point mutations alterations in the promoter/enhancer of a specific gene or the amplification of a gene.

The bottom line for this is that the mutations will either end up in the loss or an unproductive gene and its product (tumor suppressors) or an unregulated protein that is constitutively active or abnormally high levels of a protooncogenes.

One way in which harmful genes get into cells is by infection of a virus. The breakthrough in understanding of cancer at the protein/molecular level came through viral infections. The Rous Sarcoma virus was discovered by Peyton Rous and is the most intensively studies oncogene associated with a retrovirus. The genome of this virus contains relatively few genes however somewhere along the line it did pickup a major portion of the Src gene. Deletion of this gene showed that it was not vital for replication but was absolutely required for the tumor transformation and viral propagation. This portion of the gene is of course includes the catalytic domain of the tyrosine kinase and infection leads to long term

tyrosine phosphorylation and cell growth. The tyrosine kinase activity essentially short-circuits the normal signaling of the cells. It is not yet clear which target specifically leads to the transformation of the normal cells.

In addition to v-Src, many other viruses have been identified which encode part or all of protooncogenes. Nearly half of these retroviruses are protein kinases with the bulk of them being tyrosine protein kinases. The product of the v-Erb B virus (from avian erythroblastosis) is a truncated version of the epidermal growth factor. The receptor is therefore a protooncogene and could be labeled c-erb. The viral form differs from its cellular counterpart by a loss in the extracellular domain and thus the protein can not bind to the receptor. So how does the truncated lead to the transformation of these cells? This truncation allows the aggregation of the receptor and alters the conformation such that it is constitutively active, regardless of a ligand binding to an extra cellular domain. Two viruses are known to lead to the induction of cancer in humans. Burkitts lymphoma can be initiated in B-lymphocytes by the Epstein Barr virus and the leading cause of liver cancer is due to the hepatitis B virus. In the former case the promoter activity seems to have been altered for the myc gene. This is similar but distinct to the translocation activation discussed below.

Many cells exhibit chromosomal abnormalities. The translocation of chromosomes can lead to changes in the transcriptional control of genes or alter the coding region of the gene such that the protein product is now lost its normal regulation. One type of chromosomal change is found in the Philadelphia chromosome. The tumor associated with this abnormality is chronic myelogenous leukemia. In this case the chromosomes 9 and 14 are involved. The translocation between these two chromosomes leads to an altered chromosome and involves the Bcr and Abl genes. Abl is a tyrosine kinase that is normally involved in regulation of cell growth most likely through phosphorylation of various transcription factors. The new gene product is actually a fusion of the two genes, where the

regulatory domain for Abl is lost and physically replaced by Bcr. The result is an activation of the c-abl oncogene and a much increased tyrosine kinase activity.

A different type of alteration occurs in a non-viral form of Burkitts lymphoma. This is a fast growing cancer of human B-lymphocytes. In this case there is a translocation of the 8 and 14 chromosomes. A segment from the end of the large arm of the 8<sup>th</sup> chromosome breaks off and moves to chromosome 14. The reverse process moves a portion of the q arm of chromosome 14 to the 8<sup>th</sup> chromosome. The myc gene is contained in the small piece of chromosome 8 that was moved to chromosome 14. The result is the gene is now under the control of the heavy chain promoter. This is a strong promoter and in B cells is normally very active in the production of antibodies. Once the alteration has occurred the promoter now drives the production of myc. Thus unlike the last example, the alteration results in abnormally high levels of the myc gene. This mechanism is similar to enhancer insertion except that chromosomal translocation (rather than integration of a virus) is responsible for placing the protooncogenes under the influence of a foreign promoter.

Myc is a nuclear protein that interacts with other DNA proteins through leucine zippers. It is certainly a DNA binding protein and has been found to interact with the retinoblastoma protein. The increase in the level of this transcription factor seems to act to drive or force the cell into cell growth by activating genes necessary for mitosis.

Point mutations can also lead to mutagenesis. One such case is Ras. Ras is a small or low molecular weight GTP binding protein similar to the alpha subunit of the heterotrimeric G proteins. Analysis of the DNA sequencing show that two or three mutations are all that are necessary for the mutation of Ras. Highly purified Ras has led to the determination of the x-ray structure of the protein. The second and third phosphates of GTP interact specifically with two of these amino acids glycine - 12 and glutamine 61. The most common mutations of Ras occur at glycine 12 and site directed mutagenesis indicate that any change at gly12 other than proline leads to

activated Ras. The intrinsic GTPase activities of Ras are thus involved. Normally interactions of Ras with its effector leads to an increase in the hydrolysis of the gamma phosphate. The GAP activity is normally increased 100 fold but this is not the case for Ras. This leaves Ras in a GTP bound state allowing it to continue to activate its downstream effectors without regulation. Thus the Ras signal is turned on and not off. This would lead to the modification of numerous signaling pathways including the MAPKinase activity seen with growth factor activation.

### Tumor suppressor genes

Very little is known about some of the tumor suppressors. These genes code for proteins whose normal function is to turn off cell growth. A mutation in one of these genes leads to an ineffective or missing protein which is responsible for the control of cell growth and thus the cells would continue to grow without control (a.k.a. cancer). These mutations are typically recessive because unlike the oncogenic counterparts, both copies of the gene usually need to be mutated to see the effect. Because both copies of the gene need to be mutated these genes are recessive in nature.

One type of tumor suppressor is the breast cancer gene BRCA1 and BRCA2. Both of these genes are unfortunately still quite a mystery. What is mostly understood about this gene is it is only part of the development of breast cancer and is certainly one gene that cancer can be passed on generation to generation. These genes seem to account for the bulk of familial breast cancers, encompassing as many as 20% of all pre-menopausal breast cancers in this country and a substantial portion of familial ovarian cancers as well.

Two other suppressor genes are also heavily studied and more is known about their

mechanism of action. The retinoblastoma gene (Rb) was first found in eye cancers. This gene is located on chromosome 13 and it encodes a nuclear protein that is often considered the master brake of the cell. This gene is deleted or mutated in 40% of human cancers, and is involved in eye, bone, bladder, small cell lung and breast cancers. Just prior to the G1/S interface of cellular growth is a restriction point where cells rest temporarily and can undergo gene repair or cell death. This protein is regulated by phosphorylation. During the G0 or G1 phase Rb is predominately in the dephospho form. The phosphorylation increased in late G1 and S phase where cells stop growing. The protein responsible for the phosphorylation of Rb is a cyclin dependent kinase (CDK). In the unphosphorylated state Rb tightly binds a transcription factor called E2F. E2F activates genes that promote cell proliferation (cell growth). Phosphorylation of Rb reverses the interactions between Rb and E2F. Mutations of Rb typically lead to a poor binding of the transcription factor regardless of the state of phosphorylation.

Another important tumor suppressor gene is p53, a 53 kDa protein found in nearly all human cancers. This protein like the Rb protein is nuclear in location and subjected to phosphorylation. P53 seems to have three major effects 1) it acts as a transcriptional regulator of certain genes involved in cell growth. 2) It acts as a G1 check point to control for DNA damage. If excess damage occurs, the activity of p53 increases, resulting in inhibition of cell division so that DNA repair can take place. If this were not to continue, the new cells would carry on the mutation permanently and DNA damage could accumulate. Thus this role considers p53 the molecular policeman. 3) A third function of p53- one that is now under intense investigation, is that of control of the initiation of apoptosis.

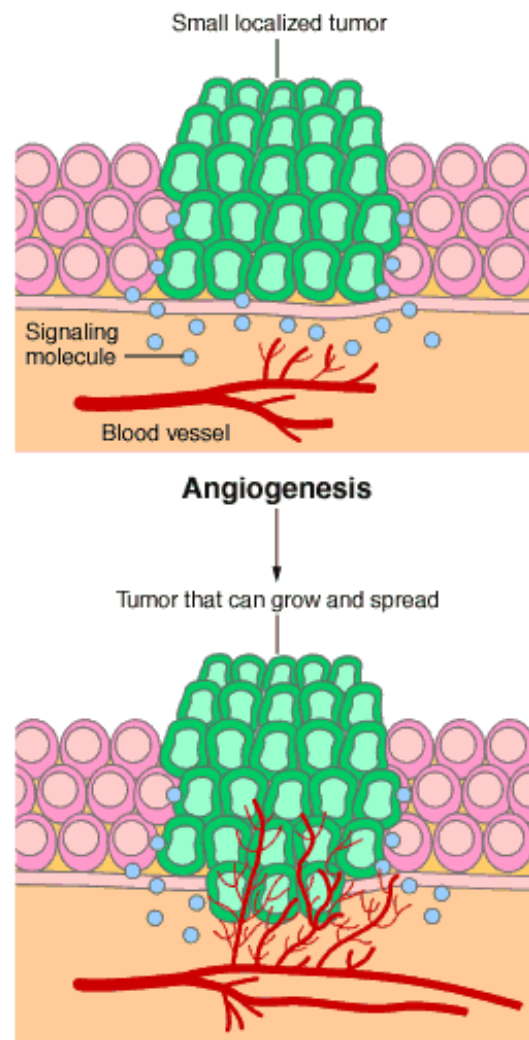
Several genes are involved in the control of cell death. Once a cell has reached a certain point of damage rather than continue to grow, it can be terminated. Myc (a transcription factor) stimulates apoptosis, whereas a different gene Bcl-2 inhibits apoptosis. P53 seems to control apoptosis by leading to an increase in the production of another gene Bax. Higher levels of Bax protein due to p53 leads to a decrease in the inhibition of apoptosis due to Bcl-2. This role of p53 is also thought to be very important in Alzheimer's, Huntington's, and Parkinson's diseases. The purpose of this function of p53 is that it hastens the cell death of potentially dangerous cells - eg, those that have been damaged by UV irradiation - which have become potentially malignant. One reason regulation of p53 is important is that if one could selectively turn on the protein in a tumor it would then send the cancerous cells into the death mode and be a vital tool in gene therapy.

Cancer presents considerable challenges to many groups of scientists. Despite many years of intensive research that has resulted in many significant discoveries, we still do not completely understand how cancer develops, nor do we know how to cure cancer. Much research will need to be done in the future so that the impact of cancer on society may be diminished.

**New Work on Fighting Cancer: Angiogenesis Inhibitors in Cancer Research** (Information from the National Cancer Institute)

One promising avenue of cancer research is the study of a group of compounds called angiogenesis inhibitors. These are drugs that block angiogenesis, the development of new blood vessels. Solid tumors cannot grow beyond the size of a pinhead (1 to 2 cubic millimeters) without inducing the formation of new blood vessels to supply the nutritional needs of the tumor. By blocking the development of new blood vessels, researchers are hoping to cut off the tumor's supply of oxygen and nutrients, and therefore its continued growth and spread to other parts of the body.

About 30 angiogenesis inhibitors are currently being tested in human trials. Most are in early phase I or II clinical (human) studies. Three are in phase III testing and the results for one are expected by the end of 1999. Phase I/II trials include a limited number of people to determine the safety, dosage, effectiveness, and side effects of a drug. In phase III trials, hundreds of people around the country are assigned at random to receive either the new treatment or the standard treatment.



As of 2000, more than 300 angiogenesis inhibitors have been discovered. There are more than 9 million cancer patients in the U.S. who could potentially benefit from antiangiogenic therapy. Several patients were reported to have experienced dramatic tumor regression.

from antiangiogenic therapy in 1988 at least 4,000 cancer patients have been enrolled in clinical trials of experimental antiangiogenic therapy

There are three main types of antiangiogenic drugs:

1. Drugs that stop new blood vessels from sprouting (true angiogenesis inhibitors)
2. Drugs that attack a tumor's established blood supply (vascular targeting agents)
3. Drugs that attack both the cancer cells as well as blood vessel cells (the double-barreled approach).

How effective are antiangiogenic therapies for cancer? Almost all antiangiogenic drugs are still experimental and undergoing clinical trials, the gold standard for determining the safety effectiveness of a new drug. The Angiogenesis Foundation estimates that more than 6,500 patients have received some form of antiangiogenic therapy through a clinical trial.

### **Background**

In normal tissue, new blood vessels are formed during tissue growth and repair, and the development of the fetus during pregnancy. In cancerous tissue, tumors cannot grow or spread (metastasize) without the development of new blood vessels. Blood vessels supply tissues with oxygen and nutrients necessary for survival and growth.

Endothelial cells, the cells that form the walls of blood vessels, are the source of new blood vessels and have a remarkable ability to divide and migrate. The creation of new blood vessels occurs by a series of sequential steps. An endothelial cell forming the wall of an existing small blood vessel (capillary) becomes activated, secretes enzymes that degrade the extracellular matrix (the surrounding tissue), invades the matrix, and begins dividing. Eventually, strings of new endothelial cells

organize into hollow tubes, creating new networks of blood vessels that make tissue growth and repair possible.

Most of the time endothelial cells lie dormant. But when needed, short bursts of blood vessel growth occur in localized parts of tissues. New capillary growth is tightly controlled by a finely tuned balance between factors that activate endothelial cell growth and those that inhibit it.

About 15 proteins are known to activate endothelial cell growth and movement, including angiogenin, epidermal growth factor, estrogen, fibroblast growth factors (acidic and basic), interleukin 8, prostaglandin E1 and E2, tumor necrosis factor, vascular endothelial growth factor (VEGF), and granulocyte colony-stimulating factor. Some of the known inhibitors of angiogenesis include angiostatin, endostatin, interferons, interleukin , interleukin 12, retinoic acid, and tissue inhibitor of metalloproteinase-1 and -2. (TIMP-1 and -2).

At a critical point in the growth of a tumor, the tumor sends out signals to the nearby endothelial cells to activate new blood vessel growth. Two endothelial growth factors, VEGF and basic fibroblast growth factor (bFGF), are expressed by many tumors and seem to be important in sustaining tumor growth.

Angiogenesis is also related to metastasis. It is generally true that tumors with higher densities of blood vessels are more likely to metastasize and are correlated with poorer clinical outcomes. Also, the shedding of cells from the primary tumor begins only after the tumor has a full network of blood vessels. In addition, both angiogenesis and metastasis require matrix metalloproteinases, enzymes that break down the surrounding tissue (the extracellular matrix), during blood vessel and tumor invasion.

### **Strategies**

Of the anti-angiogenesis drugs now in clinical trials, some were designed to target specific molecules involved in new blood vessel formation. For others, the exact mechanism of the drug is not known, but it has been shown to

be anti-angiogenic by specific laboratory tests (in the test tube or in animals).

In general, four strategies are being used by investigators to design anti-angiogenesis agents: Block the factors that stimulate the formation of blood vessels Use natural inhibitors of angiogenesis Block molecules that allow newly forming blood vessels to invade surrounding tissue Incapacitate newly dividing endothelial cells.

### ***Standard Chemotherapy Versus Angiogenesis Inhibitors***

Several differences between standard chemotherapy and anti-angiogenesis therapy result from the fact that angiogenesis inhibitors target dividing endothelial cells rather than tumor cells. Anti-angiogenic drugs are not likely to cause bone marrow suppression, gastrointestinal symptoms, or hair loss -- symptoms characteristic of standard chemotherapy treatments. Also, since anti-angiogenic drugs may not necessarily kill tumors, but rather hold them in check indefinitely, the endpoint of early clinical trials may be different than for standard therapies. Rather than looking only for tumor response, it may be appropriate to evaluate increases in survival and/or time to disease progression.

Drug resistance is a major problem with chemotherapy agents. This is because most cancer cells are genetically unstable, are more prone to mutations and are therefore likely to produce drug resistant cells. Since angiogenic drugs target normal endothelial cells which are not genetically unstable, drug resistance may not develop. So far, resistance has not been a major problem in long-term animal studies or in clinical trials.

Finally, anti-angiogenic therapy may prove useful in combination with therapy directly aimed at tumor cells. Because each therapy is aimed at a different cellular target, the hope is that the combination will prove more effective. Early trials are under way.

### **The other side of cancer – programmed cell death.**

Unlike most terms used in biomedical science, the term apoptosis is not simple to define, and this has led to some confusion and controversy. The proper pronunciation is also controversial (if in doubt try ap-a-tow'-sis). In any case there is intense current interest in this area, with a recent exponential publications concerning this subject.

The term apoptosis was coined in a now-classic paper by Kerr, Wyllie, and Currie (Brit J. Cancer 26:239) in 1972 as a means of distinguishing a morphologically distinctive form of cell death which was associated with normal physiology. Apoptosis was distinguished from necrosis, which was associated with acute injury to cells. Apoptosis is characterized by nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing. Mitochondria remain unchanged morphologically.

Apoptosis, or programmed cell death, is a physiological form of cell death that plays a critical role in the development and maintenance of multicellular organisms. There is great interest in regulation of cell death and improper control of apoptosis can contribute to several pathological conditions, including cancer. Apoptosis is activated by a variety of mechanisms only a portion of which are fully understood. Each of these signals usually results in the activation of various genes.

An important class of apoptotic genes has been identified. Caspases (cysteine aspartases) and the Bcl-2 family are some of the first discovered mammalian cell death regulators. The antiapoptotic members of the Bcl-2 family act upstream of the execution caspases somehow preventing their proteolytic processing into active killers. Two main mechanisms of action have been proposed to connect Bcl-2s to caspases. In the first one, antiapoptotic Bcl-2s would maintain cell survival by dragging caspases to intracellular membranes (probably the mitochondrial membrane) and by preventing their activation. Additionally, Bcl-2 could act by regulating the release from mitochondria of some caspases activators: cytochrome c and/or apoptosis-inducing factor.

This type of cell death is often hard to observe *in vivo* because the dying cells are rapidly phagocytosed by tissue macrophages, and this phagocytosis is clearly different from that seen in inflammation, when activated macrophages are recruited from outside the immediate area of death. The above diagram (top line) illustrates the morphological changes associated with apoptosis. The simplest way to observe this phenomenon *in vitro* is to use a cell permeant DNA-staining fluorescent dye such as Hoechst 33342, which allows a striking visualization of the chromatin condensation.

Apoptotic death can be triggered by a wide variety of stimuli, and not all cells necessarily will die in response to the same stimulus. Among the more studied death stimuli is DNA damage (by irradiation or drugs used for cancer chemotherapy), which in many cells leads to apoptotic death via a pathway dependent on p53. Some hormones such as corticosteroids lead to death in particular cells (e.g., thymocytes), although other cell types may be stimulated. Some cell types express Fas, a surface protein which initiates an intracellular death signal in response to crosslinking. In other cases cells appear to have a default death pathway which must be actively blocked by a survival factor in order to allow cell survival. When the survival factor is removed, the default apoptotic death program is triggered.

Biochemical correlates of these morphological features have emerged during the subsequent years of study of this phenomenon. The first and most dramatic is DNA fragmentation, which was described by Wyllie in 1980. When DNA from apoptotically dying cells was subjected to agarose gel electrophoresis, ladders with ~200 bp repeats were observed, corresponding histone protection in the nucleosomes of native chromatin. Subsequent pulsed field gel techniques have revealed earlier DNA cleavage patterns into larger fragments. Since even a few double stranded DNA breaks will render the cell unable to undergo mitosis successfully, such DNA fragmentation can be regarded as a biochemical definition of death. However, in

some apoptotic systems (e.g., Fas killing of tumor cells) artificially enucleated cells lacking a nucleus still die, showing that the nucleus is not always necessary for apoptotic cell death. This DNA cleavage is depicted in the central blow-up at the bottom of the diagram above.

The changes in the apoptotic cell which trigger phagocytosis by non-activated macrophages have been investigated by several groups. Macrophages appear to recognize apoptotic cells via several different recognition systems, which seem to be used preferentially by different macrophage subpopulations. There is good evidence that apoptotic cells lose the normal phospholipid asymmetry in their plasma membrane, as manifested by the exposure of normally inward-facing phosphatidyl serine on the external face of the bilayer. Macrophages can recognize this exposed lipid headgroup via an unknown receptor, triggering phagocytosis. Exposure of phosphatidyl serine on the surface of apoptotic cells is depicted in the right blow-up at the bottom of the diagram above.

Another biochemical hallmark of apoptotic death which increasingly appears general is the activation of caspases, which are cysteine proteases related to ced-3, the "death gene" of the nematode *Caenorhabditis elegans*. Caspases seem to be widely expressed in an inactive proenzyme form in most cells. Their proteolytic activity is characterized by their unusual ability to cleave proteins at aspartic acid residues, although different caspases have different fine specificities involving recognition of neighboring amino acids. Active caspases can often activate other pro-caspases, allowing initiation of a protease cascade. While several protein substrates have been shown to be cleaved by caspases during apoptotic death, the functionally important substrates are not yet clearly defined.

A critical issue is how caspases become initially activated, which seems to be an irreversible commitment towards death. It seems that aggregation of some pro-caspases (those with large pro-domains) allows them to autoactivate. Recent experiments make it clear that mitochondria are involved in one major pathway involving activation of pro-

caspase-9. Other experiments show that ligands crosslinking death receptors such as Fas trigger formation of a cytoplasmic complex in which pro-caspase-8 is aggregated and activated. In both cases these initiator caspases in turn activate a cascade of other pro-caspases leading to death.

While there is much to be learned about the molecular pathways leading to apoptotic cell

death, it is increasingly clear that cell death is a normal part of normal biological processes. This had not been appreciated until relatively recently, and our understanding of such death, and our ability to manipulate it, could allow therapeutic intervention in major diseases such as cancer, heart disease, stroke, AIDS, autoimmunity, degenerative diseases, and others.