Cancer, incurable disorder was known form the time of Mesopotamian and/or Babylonian. Various drug deliveries were developed to treat the cancer. Targeted drug deliveries are thirsty area of research. Use of Monoclonal Antibody, Peptides, protein helps in targeting to tumor cells. The surface modified liposomes are proved to be more effective for targeting to the cancer cells.
2.1 INTRODUCTION AND BACKGROUND

Cancer puzzled man kind long back and still cure is the mystery. The cancer itself can not be fatal but once it metastasize becomes dreaded for patient. Effective treatments for solid tumors, places reachable for surgery or radiotherapy are available. Chemotherapy is also effective in treatment of residual and spread disease in some tumor types, for example lymphomas. However, these treatment modalities can not cure a large number of patients due to location of the tumor, the presence of disseminated cells or recurrence of drug resistant disease. Targeted therapy might be helpful when other curative treatments fail. Tumor therapy seeking out the disseminated cells in the bloodstream and lymphatic vessels and finding the residual cells after surgery is an appealing approach gaining interest.

Imagine a healthy tissue containing thousands of cells; each cell serves the greater good, which is the continuation of a person’s life. The homeostasis has been there for peaceful and timely life or death of cells including repair and replacement whenever necessary. Communication takes place either indirectly, via exchange of messenger compounds such as hormones and growth factors, or directly, via cell-to-cell contact. Contact allows cells to respond to the “feel” of neighboring cells, via cell adhesion molecules, and to exchange messenger molecules through cell-to-cell portals called gap junctions. Cell proliferation is well defined process, as the need form new cells and in excess cell division stops. Cancer cells, the descendants of a normal cell, are uncontrolled growth due to mutation in the DNA. After the DNA has been damaged, the cell withdraws from close communication with its neighboring cells being separated from the regulatory controls of its community; hence at less infective state.

Let us say that the environment around this cell contains a promoting agent, which is a compound that stimulates cell proliferation. In response to the promoting agent, this precancerous cell divides to produce daughter cells, and these daughter cells divide to produce more daughter cells. The genetic instabilities passed down through the generations finally result in one cell that becomes capable of self stimulation known as autonomous cancer cell which no longer requires the promoting agent to stimulate its proliferation. The role of the promoting agent is made obsolete by the cell’s ability to make proteins such as growth factors that stimulate proliferation.
As the cells divide, they develop malignant characteristics, such as the ability to invade and metastasize. They also develop other characteristics that help assure survival, for example, the ability to evade the immune system, to mutate when faced with adverse conditions, and to induce the growth of new blood vessels through the process called angiogenesis. The development of these characteristics marks the third stage in carcinogenesis, the first two stages being initiation and promotion, respectively. Compared to normal cells, cancer cells have lost touch with their neighboring cells, their community purpose, and even largely with one another. They are a race of self-serving, easily adaptable cells, whose proliferation continues with the slightest provocation. They use more than their fair share of resources, live longer than their fair share of time, and produce more than their share of offspring. In short, they exhibit the two deadly characteristics of cancer: uncontrolled proliferation and uncontrolled spread.

2.1.1. Line of attack for cancer
Some points had been discussed to elevate the cancer. As the pattern of spared is not exactly same for all form of cancer but some common points have been represented.

1. Initiation of genetic instability. The every cancer cells have genetic instability within and when gets environment chances of mutation becomes more prominent.

2. Abnormal expression of genes. Malfunctioning in the expression of genes comes about defects in protein expression which increase and inhibits cancer cells progression.

3. Anomalous signal transduction. Signal transduction is the movement of a signal from outside the cell toward the cell’s nucleus, where it can stimulate proliferation or other activities. Growth factors and cell adhesion molecules (CAMs) are critical source of external signals, later are proteins that regulate the interactions of cells with their surrounding while previous are soluble molecules that bind to specific receptors on the cell’s surface and stimulate the cell’s activities. Other factors involved are the signal generation and signal transduction. The inherent property that cancer cells can produce their own growth factors, thereby allowing self-stimulation; can produce extra receptors and free radicals, which can make growth factor receptors more responsive to stimulation.
4. Abnormal cell-to-cell communication. By decreasing their contact with normal cells, cancer cells are freed to act independently. As mentioned previously, cell-to-cell communication occurs via portals between adjacent cells (gap junctions) and through cell adhesion molecules. Normal cell-to-cell communication through gap junctions maintains homeostasis and discourages cancer like behavior.

5. Induction of angiogenesis. Angiogenesis is the growth of new blood vessels toward and within tumors (or other tissues). Tumors need blood vessels to supply oxygen and nutrients, and the blood vessels created by angiogenesis provide the channel by which tumor cells metastasize to distant locations.

6. Invasion and metastasis. Tumors can spread both locally, via invasion of adjacent tissues, and distantly, via metastasis through the blood and lymph circulation. The spread of cancer, along with uncontrolled proliferation, is a central hallmark of malignancy.

7. Immune evasion. Cancer cells shield themselves from immune attack, thereby evading destruction; they can hide from immune cells by employing various camouflaging techniques or can produce immunosuppressive compounds that impair the ability of immune cells to function.

-Each of the seven clusters of procancer events is illustrated in Figure 2.01

---

Figure 2.01 Clusters of procancer events
Some of the remedy for cancer in concern to the above said point is to reduce oxidative stress which ultimately decline genetic instability. Transcription factors act as switches in nucleus to turn on gene expression and normalizing the activity of those transcription factors that control the expression of these genes. It is not possible to eliminate signal transduction but one can bring it down to normal levels as it is overexpressed in cancer cells. Normal cell-to-cell communication can be fostered by improving gap junction communication and by normalizing CAM activity. Cancer can be inhibited by blocking the release or action of angiogenic factors or by otherwise altering the local environment to inhibit tumor angiogenesis. The most dangerous things are invasion and metastasis and inhibition of these process help to cure the cancer. Invasion requires enzymatic digestion of the healthy tissue surrounding the tumor. It also requires the migration of tumor cells. Invasion can be reduced by inhibiting enzymes that digest local tissues, by protecting normal tissues from the enzymes, and by reducing the ability of tumor cells to migrate. Metastasis requires that cells detach from the primary tumor, enzymatically digest blood vessel walls to gain access to and exit from the blood circulation, and evade the immune system while in the circulation. Thus metastasis can be checked by inhibiting any one of these processes. The immune response against cancer cells can be increased by stimulating the immune system and by reducing the ability of cancer cells to evade immune attack.

2.2 METASTASIS

Metastasis is the movement of malignant cells from a primary tumor site to a distant location where they form a new tumor. Invasion and metastasis are related—to be metastasizing successfully the tumor cells have to invade the basement membrane. Most cancers do metastasize. Metastatic cells travel through the blood and lymphatic circulation systems, and the metastatic colonies they form are often more life threatening than the primary tumor. In fact, the growth of a tumor at its primary location is generally not a cause of death, except in a limited number of cancers such as those of the brain, liver, and lungs. (Brain cancer, for example, is one of the few cancers that rarely metastasize.) Since metastasis is what makes so many cancers life threatening, its inhibition is an important clinical goal.
2.2.1. Steps in Metastasis

The metastatic process is inherently inefficient. In a study of patients with kidney cancer, between 10 million and 1 billion cancer cells were released from their tumors into the bloodstream per day. In spite of this enormous release, 20 percent of the patients showed no evidence of new tumor development, even after 30 months [Glaves et al., 1988]. In animal tumors, only 0.001 percent of the released tumor cells develop into metastatic colonies [aHonn et al., 1987; bHonn et al, 1987]. The reasons for the inefficiency of the metastatic process are uncertain, but since at least five different sequential steps are required for its success, interruption of any one of them could derail the whole process. The last step in this process seems particularly sensitive to inhibition. A metastatic colony develops according to the steps listed below and illustrated in Fig. 2.02. Like many aspects of cancer, metastasis is a complicated process, and numerous factors can affect each step.

1. Cells detach from the primary tumor and invade the basement membrane surrounding blood vessels to gain access to the bloodstream, a process called intravasation.
2. Once they are circulating in the bloodstream, the migrating cells evade attack by the immune system and survive other adverse conditions.
3. The migrating cells adhere to the wall of a blood vessel at the metastatic site—the process of cell arrest.
4. The arrested cells exit the blood vessel and, by invading through the basement membrane, enter the tissues. This process is called extravasation.
5. The tumor cells proliferate and the new tumor induces angiogenesis.

Fig. 2.02 Steps in Metastasis

Studies with small video cameras suggest that at least in some cases, the majority of traveling metastatic cells come to rest in a target capillary bed and that most of these successfully exit the circulation (step four above). After this point, however, only a
small fraction begins to proliferate and form a new tumor. The majority remains
dormant, neither proliferating nor undergoing apoptosis [Morris et al., 1997]. Each of
the five steps is discussed individually below. Although they refer primarily to
metastasis via the blood circulation, metastasis through the lymphatic system can
occur by similar processes.

2.2.2. Leaving home a sticky situation
The first step in metastasis is detachment of cells from the primary tumor. Once
detached, cancer cells contact a blood vessel (usually within the tumor) and secrete or
induce the secretion of proteolytic enzymes, which digest the basement membrane.
Tumor cells then slip between the cells of the vascular lining to enter the circulation.
This process of intravasation is facilitated by the poorly developed basement
membranes and fragile capillaries produced within the tumor during angiogenesis. An
intact basement membrane is a barrier that inhibits metastasis. In some cases, broken
capillaries may allow instant access to the circulation. Cell detachment rates tend to
increase as a tumor enlarges and as it undergoes central necrosis. Other factors that
may stimulate detachment include mechanical stress, increased hydrostatic pressure
within tumors, and increased activity by various proteolytic enzymes, and decreased
expression of cell adhesion molecules on the cell's surface.

2.2.3. Migration through the Circulation
The migration of tumor cells through the circulation, second step of the metastasis.
Large percentage of the tumor cells die due to circulation force. There is considerable
evidence that the immune system plays a prominent role in inhibiting metastasis, the
issue is complex, and no simple correlation between immune status and metastatic
spread has been found. Nevertheless, numerous animal experiments do support a role
for the immune system in limiting metastasis. The immune cells that appear to be
most active in attacking migrating cancer cells are natural killer (NK) cells and
macrophages. Studies suggest that

- metastasis is more frequent in animals with immune cell deficits, including deficits
  caused by immunosuppressive drugs;
- when animals are injected with tumor tissue; metastasis is more frequent if the
  macrophages within the tumor tissue are extracted before injection;
- metastasis is less frequent in mice that have high levels of NK cells [Calabresi and
  Schein, 1993].
In addition to the immune system, other forces may destroy migrating tumor cells. These include mechanical stress as cells move through the small vessels, and toxicity caused by high oxygen levels in the blood [Tannock et al., 1992].

2.2.4. Cell Arrest a New Start
The third step in metastasis is cell arrest. Because the environment within blood vessels is inhospitable to migrating tumor cells, the cells must leave the blood vessels in order to survive. This exit is initiated by attaching to the capillary wall, referred to as cell arrest. Several factors promote cell arrest at a given location, including CAM activity, vessel damage or thinning of the basement membrane, platelet aggregation, and fibrin formation.

2.2.5. Damage to the Basement Membrane
Tumor cells adhere more efficiently to the exposed collagen of a damaged blood vessel than to normal vessel walls, and so damaged areas provide prime targets for cell arrest. Vessels can be damaged by trauma or inflammation; in fact, evidence is mounting that surgical removal of some tumors can promote tumor metastasis to existing wounds [Neuhaus et al., 1999; Abramovitch et al., 1999]. This effect may also be facilitated by the growth factors present in wound fluid. Therefore, compounds that protect the vasculature or reduce inflammation may limit cell arrest.

2.2.6. Platelet Aggregation and Fibrin Production
Platelet aggregation and fibrin production can play an important role in metastasis. Three mechanisms by which they can promote metastasis are: [aHonn et al., 1987; bHonn et al., 1987]

• Activated platelets are sticky and can act as a glue to enhance adhesion of tumor cells to the blood vessel lining.

• Platelet-secreted growth factors like platelet-derived growth factor (PDGF) can stimulate the proliferation of tumor cells and contribute to their survival within the blood circulation.

• The excessive fibrin production surrounding tumor cells enhances their stickiness and facilitates their arrest at metastatic sites.

Fibrin helps tumor cells to aggregate with each other while migrating in the blood, thereby forming a larger clump that may more easily lodge in a capillary bed. In experimental studies on mice, the efficiency of metastasis was increased when either
large numbers of tumor cells or clumps of tumor cells were injected [Hill et al., 1986]. Experimental studies have reported that migrating cells from some cancers induce platelet aggregation by modifying the prostanoid balance [Tannock et al., 1992]. It is reasonable to speculate that the mixed results of the studies are due to the limited ability of platelet aggregation inhibitors to prevent metastasis when used alone, but that when used as one part of a larger combination therapy, they may be more effective.

2.2.7. Extravasation
The fourth step in metastasis is extravasation, the movement of the metastatic colony out of the blood vessel. This is mediated by proteases and other factors in a process similar to movement into the blood vessel (step one). In both cases, the basement membrane provides an obstacle to the movement of tumor cells through the vessel wall. One of the factors that mediate extravasation is local trauma (damaged capillaries). Just as trauma may facilitate intravasation (step one) and cell arrest (step three), it may also facilitate movement out of the vessel. One study reported that when cancer cells were injected into rabbits with traumatized tissues, metastasis to the trauma site was increased 20-fold over nontraumatized tissues [Calabresi and Schhein, 1993]. Therefore, it is observed that compounds that stabilize the vasculature or basement membrane may inhibit metastasis.

2.2.8. Induction of Angiogenesis
The fifth and final step in tumor metastasis is cell proliferation and induction of angiogenesis. Without angiogenesis, a tumor is unable to grow larger than a few millimeters in diameter. Once angiogenesis is established, the tumor can seed itself in a new cycle of metastasis. The leaky vessels produced during angiogenesis allow tumor cells greater access to the circulation. Thus compounds that inhibit angiogenesis may inhibit metastasis. Similarly, any compounds discussed thus far that inhibit cell proliferation may also inhibit this last step in metastasis. Angiogenesis, the growth of new blood vessels, is needed anywhere new tissue is growing. Thus it not only occurs in benign and malignant tumors but also in wound healing, ovulation, menstruation, and pregnancy. Abnormal angiogenesis also takes place in other diseases, including rheumatoid arthritis, psoriasis, and atherosclerosis.
Researchers believe that if angiogenesis can be inhibited in cancer patients, tumor growth will be inhibited or even reversed. Although inhibition of tumor angiogenesis is a very promising anticancer therapy, it is not without challenges. The same factors and environments that drive angiogenesis during cancer also drive angiogenesis during wound healing and other normal conditions in which it occurs. Since wound healing and other normal angiogenic processes are so vital for survival, the body employs redundant mechanisms to assure that angiogenesis occurs when needed. Overriding these mechanisms to stop tumor angiogenesis is not trivial. Since angiogenesis is a normal, necessary process in healthy humans, the goal in antiangiogenic therapy is to inhibit blood vessel growth as much as possible at the tumor site while allowing it to continue as necessary elsewhere [Kerbel, 2000]. Moreover, even the use of experimental antiangiogenic drugs appears to be safe, at least in the short term. The kind of adverse effects one might expect from inhibition of normal angiogenesis have not yet been reported in rodent or human trials that studied antiangiogenic therapies [Brewer et al., 2000; Majewski et al., 1995, Majewski et al., 1996; Zhou et al., 1998; Joseph et al., 1998; Bhargava et al., 1999; Kudelka et al., 1997; Joseph et al., 1996; Eckhardt et al., 1996]. For one thing, the need for angiogenesis in most adults is small and of short duration relative to the tumor’s need. For another, the body attempts to limit aberrant angiogenesis whenever it can, and antiangiogenic therapies can assist the body in this endeavor.

2.2.9. Mechanics of Angiogenesis
Although it has been known for over a hundred years that tumors contain an abnormally dense blood vessel network, it was not until the late 60s that investigators realized tumors induce their own blood supply. In their landmark study, Folkman and Hochberg (1973) reported that tumors implanted in the eyes of rabbits grew only to a size of approximately one cubic millimeter before developing their own blood supply. Thus antiangiogenic therapies may severely limit tumor growth and metastasis. The reduction in the apoptosis leads to increase in the proliferation rate of individual cells after angiogenesis [Lu et al., 1997]. In tumors with active angiogenesis, cells do not die when they should, and therefore more cells are alive to proliferate. Angiogenesis is a complex process in which existing mature blood vessels generate sprouts, and these sprouts develop into complete new vessels. During angiogenesis, vascular cells proliferate at abnormally rapid rates. Whereas under normal circumstances capillary
Chapter 2

cells divide approximately once every 7 years, capillary cells in experimental tumors may divide once every 7 to 10 days (Scott and Harris, 1994). Angiogenesis within tumors or wounds involves at least four steps: [Denekamp, 1993; Paper, 1998].

1. Cancer cells (or adjacent tissues) secrete angiogenic factors.

2. The basement membrane surrounding a mature capillary vessel dissolves, and a bud begins to grow. The BM is a layer of specialized connective tissue that encircles capillaries and connecting (ECM), the ground substance surrounding cells and tissues and holding them in place, and the capillary itself. The BM also provides structural support to the capillary.

3. Vascular (endothelial) cells proliferate and migrate from the bud toward the angiogenic stimulus—often that means toward a low-oxygen (hypoxic) environment.

4. The sprout eventually forms a hollow tube (lumen) and joins its end with another sprout to form a new capillary vessel.

Angiogenesis is a relatively rapid process; buds can form within 48 hours of exposure to an angiogenic factor [Folkman et al., 1976]. In spite of active angiogenesis, the blood supply in a solid tumor is relatively limited compared to that of normal tissue. Tumor angiogenesis results in chaotic vessel growth, and the new vessels are surrounded by poorly developed basement membranes. Because of their abnormal basement membranes, the vessels tend to be thin-walled and leaky. Some sprouts may not fuse with others, and they become dead-end sacs. These factors, in conjunction with a lack of tumor lymph vessels, lead to the creation of pressure gradients within tumors. Pressure gradients, in turn, compress the vessels and further restrict or occlude blood flow. A lack of circulation appears to be a primary cause of the central necrosis found in many large tumors. Although this process does destroy some cancer cells, the increased pressure also limits the uptake of treatment agents and immune cells into the tumor [Jain, 1990].

At last metastasis is a five-step process consisting of cell detachment and intravasation, migration through the circulation, arrest at a new location, extravasation, and cell proliferation and angiogenesis. Successful metastasis is the deadly culmination of nearly every process we have discussed so far. At every step it seems likely that natural compounds might play a significant role in slowing down or even stopping its course.


2.2.10. Invasion

Invasion is the spread of cancer cells into adjacent tissues. Along with metastasis, the spread of cells to distant sites, it is one of the distinguishing features of malignancy. Invasion and metastasis are in fact related—cancer cells must generally invade the connective tissue surrounding blood vessels (the basement membrane) for metastasis to be successful. Tumor-induced protease (protein degrading) and glycosidase (glycoside-degrading) enzymes play key role in invasion. These enzymes provide the means by which tumors digest the extracellular matrix, thereby allowing local spread. Since invasion takes place in the ECM environment, and requires local proteolysis of the extracellular matrix, pseudopodial extension and cell migration [Liotta, 1986]. The invasive process involves genetic deregulation of the tumor cells leading to an imbalance of stimulatory and inhibitory physiologic events. This deregulation occurs in a small subset of tumor cells, > 0.05 % of circulating tumor cells establish metastasis [Liotta et al, 1974; Nicolson, 1991]. At last Cancer cells produce three types of compounds that facilitate invasion: abnormal matrix components or an abnormal mix of them that fails to bind growth factors and is easily invaded; enzymes such as collagenases and hyaluronidases that digest matrix components to allow room for invasion; and variant CD44 surface proteins that help them migrate. Natural compounds can be used to inhibit the production or action of all three, and it is reasonable to suppose natural compounds will have the greatest effect on invasion when all three are inhibited together. Active natural compounds are likely to include inhibitors of signal transduction, collagenase and hyaluronidase inhibitors, and compounds that stabilize ECM components and prevent their digestion.

2.2.11. Interaction at Cellular level

This part considers how a cell communicates with adjacent cells and how it interacts with the extracellular matrix (ECM), the ground substance that surrounds cells and tissues and holds them in place. Cell-to-cell and cell-matrix communication occurs in two ways: through cell adhesion molecules (CAMs), the surface proteins that bind cells to one another and to the ECM, and through direct cell-to-cell exchange of compounds through gap junctions, the portals that form between adjacent cells. In healthy cells, cell-to-cell and cell-matrix interactions are extremely important, as they help regulate a wide range of cellular activities, including proliferation and movement. Cancer cells, on the other hand, tend to detach from surrounding cells and from the matrix, thereby freeing themselves from signals that would restrict their
proliferation and activity. At the same time, since contact with other cells and the matrix also provides “do not die” signals, cancer cells have evolved ways to mimic these signals, chiefly through growth factor production and increased signal transduction, thereby assuring they can survive as detached cells. Cell-to-cell communication is a dynamic, complex process. Cells do not exist distinct from their environment. Intercellular communication and cell-matrix interactions are vital processes that link a cell to its environment and so play an important role in the life of a healthy cell. Cancer cells commonly exhibit aberrant forms of cell-cell and cell-matrix communication, and this aberrant communication is one factor that allows them to act independently and malignantly.

### 2.2.12. Gap junctions

The last form of cell-to-cell communication discussed is intercellular communication through gap junctions. Such communication almost universally maintains tissue health and inhibits cancer progression. Gap junctions, which are portal structures between adjacent cells (see Figure 2.01), allow cells to exchange ions and small molecules directly, including ions and molecules used in signal transduction. Gap junctions can also transfer toxins, thus allowing a toxin to be distributed over many cells; such spreading and dilution of a toxin can help prevent cell death. Gap junctions themselves are comprised of proteins called connexins. We can envision connexins as short rods that, when arranged in a circle, form a tube. The tube in one cell then links up with the tube in an adjacent cell, thereby forming the gap junction. As discussed earlier, when cells become cancerous, they detach from neighboring cells. This occurs in part through downregulation of connexin genes. In fact, many if not all tumor-promoting agents reduce gap junction communication [Yamasaki et al., 1996]. Normal gap junction communication has the opposite effect of tumor promoting agents—it decreases malignant behavior. Furthermore, restoring gap junction communication between cancer cells (by gene transfection) has been reported to cause them to behave more like normal cells [Ruch, 1994]. Therefore, in recent years connexin genes have become viewed as a family of tumor suppressor genes.

*Gap junctions can exchange compounds of molecular weight below about 1,000 grams/mole.*
2.2.13. Cell Adhesion Molecules and their Receptor

Intra-cellular and cell-extracellular matrix (ECM) interactions are of great significance in many biological processes of growth, apoptosis, differentiation and cell migration, as well as cancer cell invasion and dissemination. These functions are mediated by many cell adhesion molecules and cell surface receptors. Several families of adhesion molecules have been identified and their synthesis and expression on the cell membrane studied in relation to the invasive and metastatic phenotype. Results of studies on tumor metastasis have demonstrated that cell adhesion plays an important role in various steps of the metastatic cascade and that dysregulation of adhesion mechanisms contributes to the formation of metastasis [Miyasaka, 1995]. On the one hand, certain adhesive interactions may diminish the metastatic process. For example, adhesion molecules that promote homotypic cell adhesion among homotypic tumor cells in a primary tumor site will likely diminish the metastatic potential. In that context, downregulation of these adhesion molecules has been shown to correlate with a higher propensity of tumor cells to detach from the primary site and to spread. On the other hand, up regulation of other adhesion molecules correlates with a higher potential of tumor cells to metastasize. Preferential adhesion of metastatic tumor cells to vascular endothelial cells of certain organs has been demonstrated in experimental tumors and it may be explained in part by the organ-specific expression pattern of some adhesion molecules [Zetter, 1993].

Cell adhesion molecules are specialized proteins located on the outside of the plasma membrane. Due to recent advances in laboratory techniques, research on CAMs has flourished and the pivotal role they play is understood more completely. Through interactions with the ECM and other cells, CAMs regulate proliferation, architecture, cell migration, differentiation, apoptosis, angiogenesis, and invasion [Roth, 1994; Agrez and Bates, 1994; Pignatelli and Vessey, 1994; Juliano and Varner, 1993; Brooks, 1996]. It is generalized that CAMs as the fingers of a cell, but instead of 10 fingers, cells have many hundreds, each lasting only for a few hours. Depending on the type of CAM, they generally have three functions. First, they grasp molecules on other cells or on the matrix. In some cases, this grasping helps a cell move and in others, helps it stay in place. Second, they send signals to the nucleus telling it what they feel. Third, they, or associated proteins, receive signals back from the nucleus that alters CAM behavior.
The integrins, laminin, fibronectin, vitronectin, cadherins, selectins, and the immunoglobulin super-family of adhesion molecules make family of CAMs (Fig. 2.03). Although each of these CAMs plays complex roles, in general the cadherins hold tissues tightly together and the other three help in cell migration, especially that of immune cells. One other CAM family, the CD44 surface molecule, also helps cells to migrate. Because all of these play a role in immune cell migration, it is useful to outline how these CAMs allow leukocytes (immune cells) to attach to and migrate through a vessel wall during an immune response. After floating in the bloodstream and arriving at a target location such as an infected area, leukocytes must attach to the inner vascular wall, and then slip through the vascular tissue. This three-part process consists of (a) transient interactions between selectins and integrins on the leukocytes and vascular cells, which pull leukocytes from the circulation and initiate their rolling along the vascular wall; (b) interactions with chemotactic proteins secreted by vascular cells, which cause leukocytes to creep along the vascular wall toward the site of infection; and (c) interactions between the immunoglobulin super-family of adhesion molecules (ICAMs, VCAMs, PECAMs) and integrins that induce the cells to arrest (stop at a particular place on the cell wall), spread, and finally migrate through the vascular wall.

The process is of interest not only because it is important in the immune response, but also because it is likely that blood-borne tumor cells bind, creep, and stop at a metastatic site in a similar fashion. In other words, integrins, selectins, and the immunoglobulin super-family of adhesion molecules all likely play a role in cancer metastasis. The exact role they play in cancer is complex; but in general, these CAMs tend to be overexpressed in cancer cells, and the natural compounds discussed here tend to inhibit their expression or activity and thus cancer metastasis.
2.2.14. Integrins
Integrins bind to a number of ECM proteins, including collagen, laminin, and fibronectin, and they are receptors for certain selectins and immunoglobulin superfamily adhesion molecules, including intercellular adhesion molecules (ICAMs). Like other CAMs, the intracellular root of integrins is attached to the cell’s internal (actin) cytoskeleton. Through these connections, integrins and other CAMs can affect signal transduction and cellular structure. Integrins are the most ubiquitous and versatile of all adhesion receptors [Pignatelli and Stamp, 1995]. Of all the CAMs, they are the ones most responsible for anchoring cells to the ECM. Their expression and regulation is controlled in a complex, dynamic fashion involving feedback from cell-cell, cell matrix, and cell-growth factor interactions. At different stages in a cell’s life and/or in response to changes in their microenvironment, various integrins are expressed to allow necessary growth, architectural changes, movement, and function. Quantitative and qualitative changes in integrin expression have been observed in a large number of human tumors [Pignatelli and Stamp, 1995]. Numerous integrins can be affected, and the effects may be complex. In some cases, integrin binding can prevent cancer progression by keeping cells in contact with the matrix. Indeed, in some highly metastatic human tumors, the synthesis of specific integrins is reduced
In general, however, integrins tend to be overexpressed in cancer cells, a situation that leads to increased arrest of the cells on the vascular lining during metastasis [Miloszewksa et al., 1998; Carreiras et al., 1999]. For example, human prostate cancer cells express a large number of integrins, and blocking these integrins reduces their invasion through vessel membranes in vitro [Trikha et al., 1996]. In fact, highly metastatic cells tend to adhere more easily to the vascular lining than low or non-metastatic cells—an effect due in part to excess integrin expression [Koike et al., 1995]. Because of the complexities in the function and activity of integrins, some uncertainties exist about the potential role for integrin manipulation in cancer treatment. Nevertheless, inhibition of integrin expression does seem to inhibit cancer progression. Indeed, a number of drug delivery are in clinical trials which have ability to inhibit integrin expression, and their actions generally lead to antiproliferation, antiinvasion, or antimitastatic effects, or all three.

Integrins are a widely expressed family of heterodimers comprised of a common β chain non-covalently associated with a variety of α chains that confer ligand specificity. The β1 subfamily contains six heterodimers (VLA-1 to VLA-6) that serve as receptors for ECM components such as laminin, collagen and fibronectin. VLA-4 also acts as a cell–cell adhesion molecule by recognizing the vascular cell adhesion molecule 1 (VCAM-1). The β 2 subfamily, also named leukocyte integrins because of their preferential expression site, have three members: LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150, 95 (CD11c/CD18). The presence of multiple integrins on the cell surface enables the cell to recognize its own ECM and those secreted by other cells. This recognition information is responsible for the positional information which cells need for anchorage, polarity, differentiation, and migration. Moreover, the ability of tumor cells to invade also seems to depend on the interaction of integrins with their ligands [Hynes, 1992]. Abnormal expression of β1 integrins has been associated with the increased metastatic ability of tumor cells [Chan BMC et al., 1991]. In fact, expression of VLA-2 and VLA-3 has been associated with the metastatic behavior in some neoplasms while VLA-4 is mainly expressed on myeloid and lymphoid cells and in some neoplastic cells, where it can act as the receptor for fibronectin and for VCAM-1. A positive correlation between VLA-4 expression and the metastatic potential was observed in breast cancer cell lines. However, an inverse
correlation was found between VLA-5 expression and motility and tumorigenesis in virus-transformed murine cell lines [Plantefaber and Hynes, 1989]. Considering this dual role, it seems that cell–matrix adhesion may be required for cell locomotion in certain cases, whereas it limits cell motility and growth in others [Behrens et al., 1992]. Integrins, in a similar manner as other cell adhesion molecules, are expressed on the cell surface in different states of activation. This fact implies that expression of a particular adhesion molecule does not necessarily ensure that the cell adheres to its ligand [Diamond and Springer, 1994].

In addition to their structural functions, integrins mediate signalling from the extracellular space into the cell through integrin-associated signalling and adaptor molecules such as FAK (focal adhesion kinase), ILK (integrin-linked kinase), PINCH (particularly interesting new cysteine - histidine rich protein) and Nck2 (non-catalytic (region of) tyrosine kinase adaptor protein 2). Via these molecules, integrin signalling tightly and cooperatively interacts with receptor tyrosine kinase signalling to regulate survival, proliferation and cell shape as well as polarity, adhesion, migration and differentiation. In tumor cells of diverse origin like breast, colon or skin, the function and regulation of these molecules is partly disturbed and thus might contribute to the malignant phenotype and pre-existent and acquired multidrug resistance [Stephanie et al., 2006]. Brakebusch et al., (2002) reviewed and effect of integrins signaling in invasion and metastasis.

2.2.15. Selectins
The selectin family consists of three members, named for the cells on which they were first discovered. L-selectin is expressed on leukocytes and attaches to activated endothelial cells. E-selectin is produced by endothelial cells and attaches to leukocytes. P-selectin is preformed, and then stored in platelets and some endothelial cells; it attaches to the same cells as E-selectin. The junctions produced by selectin binding are relatively weak. Hence E-selectin plays a role in leukocyte rolling. When overexpressed in cancer, selectins, like integrins, tend to help metastatic cancer cells arrest at locations in the vasculature, where they can then begin new colonies. Each selectin molecule contains strings of proteins called lectins that are devoid of sugar chains. These proteins bind sugars (carbohydrates) on adjacent cells in a “lock and key” fashion. They can also bind with integrins in a similar fashion. In addition, these proteins can bind to free sugars. If all the sugar-binding sites are filled with free
sugars, the selectin is not able to bind to other cells. Based on recent studies, it may be possible to saturate the proteins through oral administration of certain sugars. In regard to cancer, this would reduce the initial binding of tumor cells to the vascular wall as they travel through the circulation. One sugar that has been studied is modified citrus pectin. Oral administration of modified citrus pectin inhibited metastasis of melanoma cells in mice and prostate cancer cells in rats [Pienta et al., 1995; Inohara and Raz., 1994; Platt and Raz., 1992]. Moreover, given orally to mice at a daily dose of 310 and 620 milligrams it reduced the growth of transplanted colon cancer cells by 38 and 70 percent, respectively [Hayashi et al., 2000].

2.2.16. Immunoglobulin Superfamily of Adhesion Molecules
The immunoglobulin superfamily comprises a wide variety of molecules that share the basic architecture of the immunoglobulins. Few members of this superfamily have been implicated in tumor metastasis. The most studied groups of immunoglobulin adhesion molecules are N-CAMs (found in nervous tissue), intercellular cell adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), and platelet endothelial cell adhesion molecules (PECAMs). All of these play a role in assisting immune cells and cancer cells to arrest at a target location on the vascular wall. ICAM-1 is a cell surface adhesion glycoprotein that is constitutively expressed by endothelial cells and by some leukocytes. ICAM-1 surface expression can be induced in many other cell types through the action of inflammatory cytokines. Cells expressing ICAM-1 can support adhesion of leukocytes by specific interaction with integrins of the β2 subfamily. ICAM-1 expression has been correlated with an increased risk of metastasis in some neoplastic cells [Johnson et al., 1989]. However, the identity of the endothelial ligand for ICAM-1 is still unclear. A circulating form of ICAM-1 has been found in increased levels in patients with cancer [Seth et al., 1991]. Nevertheless, it remains to be determined whether these increased levels reflect merely a greater tumor burden, or whether they contribute directly to the progression of malignancies. The vascular cell adhesion molecule 1 (VCAM-1) is an endothelial ligand for the VLA-4 integrin. Its expression is induced in endothelial cells by cytokine activation. Melanoma cell lines have been shown to adhere to activated umbilical vein endothelial cells via LFA-4/VCAM-1 interaction. This has been hypothesized to be partly responsible for retention of leukemia cells in the bone marrow and metastasis of lymphoma to this territory. However, its role in favoring
spread in malignancies from other origins is unclear. In that context, decreased levels of VCAM-1 have been associated with metastatic behavior of some tumor cells, thus suggesting that its cellular expression may be important in preventing metastasis by cell-cell adherence [Denton et al., 1992]. The neural cell adhesion molecule (NCAM) is expressed frequently in bile duct cancer, and a significant correlation has been found between its expression and perineural invasion [Seki et al., 1993]. This fact suggests that bile duct carcinoma cells invade into the perineural space by recognizing NCAM receptor expressed on neural cells. Carcinoembryonic antigen (CEA), a clinically useful marker for tumor recurrence in colorectal cancer, also belongs to the immunoglobulin superfamily. It has been shown that CEA may act as an intercellular adhesion molecule on the surface of colon carcinoma cells [Benchimol et al., 1989]. However, it is unknown whether dysregulation of this molecule actually leads to detachment of tumor cells from the primary tumor and their spreading to distant tissues.

\textbf{2.2.17. Cadherins}

In contrast to the three families of CAMs discussed above that play a role in immune cell migration, cadherins play a role in maintaining the structure of tissues. Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion in normal tissue as well as in various tumors [Takeichi, 1990; Takeichi, 1991]. So far, more than 20 classes of cadherins have been identified. Three of them, E-cadherin (epithelial cells), N-cadherin (nerve cells) and P-cadherin (placental cells) share a common basic structure but display a unique tissue distribution pattern. They bind to other cadherins on adjacent cells in a zipper like fashion, forming a tight bond between cells, the tightest formed by any cell adhesion molecule. E-cadherin is the most extensively studied with regard to its role in cancer invasion. Cancer cells, being relatively poorly differentiated, commonly display a decreased number of E-cadherin molecules or a decrease or malfunction in catenin molecules, which are the proteins that attach cadherins to the intracellular actin cytoskeleton. In cancer metastasis, the detachment of cells from the primary tumor is an initial step, which is caused by a disruption of mutual cell connections. Reduced E-cadherin expression or function allows cancer cells to detach from adjacent cells. Not surprisingly then, E-cadherin activity appears to act reliably and consistently to suppress invasion of cancer cells. Moreover, under expression of E-cadherin has been associated with poor prognosis,
Chapter 2
decreased differentiation, and increased tumor invasion and metastasis in a wide range of human tumors [Bracke et al., 1996; Heimann et al., 2000]. Besides cell adhesion functions, loss of cadherin activities may have other consequences. Abrogation of E-cadherin up regulates synthesis of ECM-degrading proteases in tumor cells [Frixen and Nagamine, 1993]. Considering that penetration of surrounding tissues is the hallmark of invasion, it is tempting to speculate that such a process may be initiated by dysregulation of some adhesion molecules [Miyasaka et al., 1995]. Consequently, stimulation of E-cadherin expression or function may present an ideal target for cancer therapy.

2.2.18. Laminin

Laminin was first described in 1979 as a large (\(M_r = 800\,000\)) basement membrane-derived non-collagenous glycoprotein, heterotrimer of three subunits, \(\alpha\), \(\beta\) and \(\gamma\) held together by disulphide bonds to form a shape of a cross (Fig 2.04) [Aumailley and Smyth, 1998; Engvall and Wewer, 1996; Malinda and Kleinman, 1996; Engbring and Kleinman, 2003]. Important biological functions have been identified for several of the isoforms. At present, five \(\alpha\), three \(\beta\), and three \(\gamma\) chains have been described and by combination they assemble to form over 14 laminin isoforms [Patarroyo et al., 2002] that have different tissue distributions and development functions [Malinda and Kleinman, 1996; Engbring and Kleinman, 2003] (table 2.01) which are assembled into at least 12 distinct isoforms of laminin, with laminin-1 being composed of \(\alpha 1\beta 1\gamma 1\) chains, laminin-2 composed of \(\alpha 2\beta 1\gamma 1\) chains, etc. These laminin isoforms have different tissue- and development-specific localizations, suggesting important functions. For example, laminins-8 and -10 (\(\alpha 4\beta 1\gamma 1\) and \(\alpha 5\beta 1\gamma 1\)) have been described in the endothelial cell basement membrane, while laminin-2 is localized in muscle basement membrane. Laminin is the first basement membrane component appearing during the early stages of embryonic development, and displays a remarkable repertoire of biological functions [Ekblom et al., 2003; Ryan and Chridtiano, 1996]. Laminin is essential for basement membrane assembly [Malinda and Kleinman, 1996; Engbring and Kleinman, 2003; Nomizu et al., 1998] and angiogenesis [Malinda et al., 1999; Kibbey et al., 1992], induces neurite outgrowth [Weeks et al., 1990; Weeks et al., 1991], affects gene expression [Weeks et al., 1998; Reich et al., 1995; Kubota et al., 1992; Brushkin-Harav and Littauer, 1998] and is involved in cell proliferation [Kubota et al., 1992], migration [Aznavoorian et al.,

29
1990; Colucci et al., 1996] and differentiation [Rozzo et al., 1993]. Biochemical dissection related some of the laminin functions to specific parts of the glycoprotein. It appears that different parts in the molecule have different effects on cells. Some of these parts are cryptic and interact with cells only after cleavage of laminin by proteases [Engvall and Werwr, 1996; Nomizu et al., 1998]. In vitro, most structural and functional studies have been performed with laminin-1 (α1β1γ1), the main component of Matrigel, which is an extract of basement membrane derived from a murine tumor, and its components are identical, both chemically and immunologically, to authentic basement membrane components [Patarroyo et al., 2002].

Table 2.01 Isoforms of the laminin family

<table>
<thead>
<tr>
<th>Name</th>
<th>Chain composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin-1</td>
<td>α1β1γ1</td>
</tr>
<tr>
<td>Laminin-2</td>
<td>α2β1γ1</td>
</tr>
<tr>
<td>Laminin-3</td>
<td>α1β2γ1</td>
</tr>
<tr>
<td>Laminin-4</td>
<td>α2β2γ1</td>
</tr>
<tr>
<td>Laminin-5</td>
<td>α3β3γ2</td>
</tr>
<tr>
<td>Laminin-6</td>
<td>α3β1γ1</td>
</tr>
<tr>
<td>Laminin-7</td>
<td>α3β2γ1</td>
</tr>
<tr>
<td>Laminin-8</td>
<td>α4β1γ1</td>
</tr>
<tr>
<td>Laminin-9</td>
<td>α4β2γ1</td>
</tr>
<tr>
<td>Laminin-10</td>
<td>α5β1γ1</td>
</tr>
<tr>
<td>Laminin-11</td>
<td>α5β2γ1</td>
</tr>
<tr>
<td>Laminin-12</td>
<td>α2β1γ3</td>
</tr>
<tr>
<td>Laminin-14</td>
<td>α4β2γ3</td>
</tr>
<tr>
<td>Laminin-15</td>
<td>α5β2γ3</td>
</tr>
</tbody>
</table>

Fig 2.04 Active sites on laminin 1
Laminin promotes tumor progression

Metastatic spread of cancer continues to be the greatest challenge to cancer cure. At the core of the process lie the changing adhesive preferences of the tumor cells, which determine their interactions with other cells and with the extracellular matrices, mainly in attachment and degradation processes [Chambers and Matrisian, 1997; Ménard et al., 1997]. Basement membranes are lost or penetrated by tumor cells during invasion and metastasis, and discontinuities were shown in basement membranes of malignant tumors but not in those of their benign counterparts. Therefore, laminin-cell interactions in tumors are different from that of normal tissue and are more tumorigenic and more malignant than either the parental cells or fibronectin adhesion-selected cells [Ekblom et al., 2003]. In general, epithelial tumors display a laminin chain composition similar to that of their tissue of origin [Patarroyo et al., 2002], but the expression of laminin receptors is altered in cancer [Patarroyo et al., 2002; Ménard et al., 1998]. More than 20 cell surface receptors have been identified for laminin, including integrins, a 32/67 kDa protein, proteoglycans, sulphatides, gangliosides, amyloid precursor protein, lectins, and Galactosyltransferases. The interaction of cancer cells with laminin was identified as a key event in tumor invasion and metastasis [Malinda and Kleinman, 1996; Engbring and Kleinman, 2003]. Invading tumor cells attach to laminin and the interaction increases the metastatic potential of tumor cells. In tumors, laminin is produced by cells in the extracellular matrix, and by tumor cells [Alitalo et al., 1981; Siwek et al., 1992; Stenback and Wasenius, 1986].

Laminin promotes tumor dissemination by several mechanisms. One of the mechanisms by which laminin contributes to the metastatic spread is induction of tumor cell proliferation. Laminin-1-adherent cells showed increased proliferative activity and reduced apoptosis in comparison with the laminin-1-nonadherent cells [Ho Kim et al., 1999]. In addition, it was shown that laminin is chemotactic and haptotactic for tumor cells [Aznavoorian et al., 1990], therefore involved in tumor cell migration. Furthermore, laminin promotes tumor cell invasion by induction of proteases that degrade various components of the extracellular matrix. In certain metastatic cells, but not in normal cells, laminin induces an increase in matrix metalloproteinase-2 (MMP-2) activity [Reich et al., 1995], which has a key role in invasion and metastasis [Nabeshima et al., 2000; Yu et al., 1996]. Indirectly, laminin
and more than 20 peptides derived from the glycoprotein contribute to tumor dissemination by promoting angiogenesis. The role of the different laminin isoforms in tumor invasion, angiogenesis and metastasis was reviewed by Patarroyo et al. (2002). It was found that laminin peptides have different malignant properties [Yamamura et al., 1993]. For example, the YIGSR (amino acids 828–933 on the β1 chain) sequence of the β1 chain in laminin-1 promotes tumor cell attachment and migration, and when injected together with melanoma cells into a mouse, it inhibits melanoma lung cell colonization, solid tumor growth, and angiogenesis via the 32/67 kDa receptor. SIKVAV (amino acids 2099–2104 on the α1 chain), of α1 chain increases angiogenesis, tumor growth and metastasis when injected together with melanoma cells into a mouse [Malinda et al., 1999; Yamamura et al., 1993]. Another sequences, VAYI (amino acids 127–130 on the α1 chain) and the homologous peptide YVRL (amino acids 144–147 on the γ1 chain) promote angiogenesis and tumor growth via integrins αvβ3 and α5β1. LQVQLSIR (amino acids 2623–2630 on the α1 chain) promotes metastasis. This sequence recognizes cell surface proteoglycans, including syndecan-1 and a chondroitin–heparan sulphate proteoglycan on melanoma cells. Thus, multiple active sequences on laminin have been found that promote the malignant phenotype and regulate

**Laminin receptors**

The biological effects of laminin are mediated by laminin receptors that are divided into two major groups: integrin and non-integrin receptors (table 2.02) [Patarroyo et al., 2002; Menard et al., 1997; Sasaki et al., 2004; Hemler, 1993]. Insufficient data exist regarding the roles of both families of receptors in mediating the various effects of laminin [Menard et al., 1998; Engvall and Wewer, 1996]

### Table 2.02 Laminin receptors and their additional ligands

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrins</td>
<td></td>
</tr>
<tr>
<td>α1β1</td>
<td>collagen (I,II,IV), laminin</td>
</tr>
<tr>
<td>α2β1</td>
<td>collagen (I,II,IV), laminin, chondroadherin</td>
</tr>
<tr>
<td>α3β1</td>
<td>fibronectin, collagen (I), laminin, nidogen, epiligrin, perlecan</td>
</tr>
<tr>
<td>α6β1</td>
<td>laminin</td>
</tr>
<tr>
<td>α6β4</td>
<td>laminin</td>
</tr>
<tr>
<td>α7β1</td>
<td>laminin</td>
</tr>
<tr>
<td>67kD laminin receptor</td>
<td>laminin</td>
</tr>
<tr>
<td>Dystroglycan</td>
<td>laminin, agrin, perlecan</td>
</tr>
<tr>
<td>Heparan sulphate</td>
<td>laminin, collagen XVIII</td>
</tr>
</tbody>
</table>
67-kDa Laminin Receptor

Several different laminin-binding cell surface proteins have recently been described. Among them, a 67-kDa, non-integrin receptor, high affinity, laminin-binding protein has been associated with the invasive and metastatic phenotype of cancer cells [Castronovo, 1993; Ménard et al., 1997]. It has been demonstrated that highly metastatic cancer cells express significantly more 67-kDa laminin receptor on their surface than their much less or benign counterparts. A highly conserved 37kD protein is the precursor of the receptor [Romanov et al., 1995; Rao et al., 1989; Butô et al., 1998], but the exact manner by which it configures its mature form is not clear. It was suggested that acylation followed by homo- or heterodimerization of the acylated 37kD precursor, by non-covalent bonds, forms the mature 67kD laminin receptor [Rao et al., 1989; Butô et al., 1998].

Two laminin binding sites were identified on the 67kD laminin receptor. The first is called G peptide (amino acids 161-180) [Castronovo et al., 1991; Magnifico et al., 1996; Taraboletti et al., 1993], and the second is at the carboxy terminal (amino acids 205-229), and binds to the peptide YIGSR on β1 chain of laminin [Landowski et al., 1995; Gloe et al., 1999]. The 67kD laminin receptor therefore recognizes various binding sites on laminin that are different from the sites recognized by integrins [Landowski et al., 1995; Canfield and Khakoo, 1999], allowing for higher overall binding affinity, but also a range of binding and signaling options. The receptor is involved in several physiological processes such as implantation [Zhang et al., 2000], invasive phenotype of trophoblastic tissue [Van Den Brule et al., 1994], angiogenesis [McKenna et al., 2001; Donaldson et al., 2000], T-cell biology [Canfield and Khakoo, 1999] and shear stress-dependent endothelial nitric oxide synthase expression [Gole et al., 1999]. Expression of the receptor has been shown to be up-regulated in neoplastic cells compared to their normal counterparts and directly correlates with an enhanced invasive and metastatic potential in numerous malignancies [Satoh et al., 1999; Sanjuán et al., 1996]. The receptor has been implicated in laminin-induced tumor cell attachment [Satoh et al., 1999; Mafune and Ravikumar, 1992] and migration [Vande Broek et al., 2001], as well as in tumor angiogenesis [Tanaka et al., 2000], growth, invasion and metastasis [Satoh et al., 1999; Mafune et al., 1992].
Integrin receptor

Integrins are a large family of cell receptors for extracellular matrix proteins and ligands on other cells (table 2.02). Integrins are heterodimeric combination of various \( \alpha \)-subunits with various \( \beta \)-subunits [Engvall and Wewer, 1996; Hemler, 1993]. By having multiple integrins as receptors for common extracellular matrix proteins, cells have the flexibility to interact with different affinities at the same ligand site and at different sites within the same ligand. The ligand specificity for different integrins can be altered depending on the type of divalent cation present, the surrounding lipid environment and various cell-specific factors. Inside the cell, the short cytoplasmic domains of integrins associate with various cytoskeletal proteins that mediate integrin signal transduction [Hemler, 1993]. At least eight integrins bind laminin; some of them bind additional extracellular matrix components as well, and cellular response depends on the sum of integrin-extracellular matrix interactions [Engvall and Wewer, 1996]. Integrins recognize mainly laminin \( \alpha \) chains and hence determine cell adhesion and response to laminin isoforms. Although some functions may be common to all laminin variants, other may be unique and isoform-specific, depending on the tissue or organ in which they are abundant [Patarroyo et al., 2002].

67kD laminin receptor and integrins

Integrins and the 67kD laminin receptor act together in transducing laminin effects. Limited data exist regarding the roles of the different receptors in mediating specific laminin effects. There are studies that indicate an association between the 67kD laminin receptor and the \( \alpha 6 \) integrin subunit, which is a part of the laminin-binding integrins \( \alpha 6\beta 4 \) and \( \alpha 6\beta 1 \) [Belkin and Stepp, 2000]. It was found that activation of human T lymphocytes induces an increase in both 67kD laminin receptor and \( \alpha 6\beta 1 \) integrin expression, and that the two receptors mediate avid cellular adherence to laminin [Canfield et al., 1999]. The 67kD laminin receptor and the \( \alpha 6\beta 1 \) integrin were shown to be co-expressed and co-regulated in small-cell lung carcinoma cell lines, and their expression correlated with ability to adhere to laminin [Pellegrini et al., 1994]. An additional study showed increased expression of the \( \alpha 6 \) integrin subunit and of the 67kD laminin receptor in pancreatic adenocarcinoma specimens, compared with normal pancreatic tissue from the same patient, indicating co-regulation of the receptors [Halatsch, 1997]. As opposed to the above report, differential expression of the \( \alpha 6 \) integrin subunit and the 67kD laminin receptor was seen in human
hepatocellular carcinoma. Although higher expression of both the \( \alpha 6 \) integrin subunit and the 67kD laminin receptor was found in tumor specimens compared to normal tissues from the same patient, the increase in \( \alpha 6 \) integrin subunit expression was more pronounced than that of the 67kD laminin receptor, indicating different regulation of receptor expression [Ozaki et al., 1998]. An in vitro study on human vulvar epidermoid carcinoma it was found that co-regulation and physical association of the \( \alpha 6 \) integrin subunit and the 67kD laminin receptor [Magnifico et al., 1996]. Integrins bind laminin at different sites than the 67kD laminin receptor, which may lead to higher laminin-binding affinity. Some investigators suggested that the 67kD laminin receptor is just a co-factor for laminin-integrin interactions [Magnifico et al., 1996], but other reports indicate that the 67kD laminin receptor may have additional functions [Gauczynski et al., 2001]. The 67kD laminin receptor does not co-localize with \( \alpha 6 \) integrin subunit in neuroblastoma cell line [Gauczynski et al., 2001]. It was found that A375SM melanoma cells express two alternatively spliced isoforms of the \( \alpha 6 \) integrin subunit, \( \alpha 6A \) and \( \alpha 6B \). However, cells expressing reduced 67kD laminin receptor showed a significantly reduced mRNA level of the \( \alpha 6B \) integrin subunit isoform, with no significant change in \( \alpha 6A \) isoform mRNA level. Thus, the \( \alpha 6B \) is the important isoform in the concept of co-regulation with the 67kD laminin receptor in the A375SM melanoma cell line [Givant-Horwitz et al., 2004]. 67kD laminin receptor mRNA and protein expression was found to be independent of that of the \( \alpha 6 \) integrin subunit in both solid tumors and effusions of serous ovarian carcinoma. Expression of the 67kD laminin receptor was detected in the majority of specimens, at all anatomic sites, and did not correlate with clinico-pathological parameters or survival. In contrast, loss of \( \alpha 6 \) integrin subunit expression predicted better overall survival [Givant-Horwitz et al., 2003].

2.2.19. Fibronectin

Fibronectin (FN) is a major constituent of ECM and is a multifunctional glycoprotein that promotes the attachment, spreading and migration of various cells [Pierschbacher and Rousslati, 1982]. Fibronectin contains at least two major domains that support cell adhesion [Kornblith et al., 1989]. One is the central cell binding domain containing the adhesive recognition tetrapeptide, Arg-Gly-Asp-Ser, which is recognized by a variety of cell types including fibronectins that support cell adhesion and is recognized by a variety of cell types via the integrin \( \alpha 6\beta 1 \). The second,
originally identified by its ability to support melanoma cell adhesion, is located in the alternatively spliced type III connecting segment (IIICS). A dominant cell type specific adhesion site within the IIICS has been localized to a peptide designated as CS1 comprising its amino terminal 25 residues. The receptor for CS1 is the integrin α₄β₁. A peptide comprising the carboxyl terminal 8 amino acids of CS1, EILDVPST, was found to support melanoma cell spreading, while all peptides without this sequence had little or no activity. Two smaller overlapping pentapeptides, EILDV and LDVPS, were also active, where as EILEV, containing a conservative substitution of GLU for ASP, was inactive. The reports suggested that the minimum sequence for cell adhesion activity is Leu-Asp-Val, the tripeptide sequence common to both active peptides. This prediction was confirmed by the observed ability of the Leu-Asp-Val peptide itself to block spreading on fibronectin, whereas Leu-Glu-Val was inactive. Interspecies amino acids sequence comparison also supports the importance of the LDV sequence, since it is completely conserved in the IIICS regions of human, rat, bovine, and avian fibronectins.

The principal region of the human plasma fibronectin (FN)' molecule mediating the adhesion of melanoma cells is the alternatively spliced type III connecting segment, IIICS [Hynes and Yamada, 1982; c Humphries et al., 1988]. By examining a series of overlapping synthetic peptides spanning the entire IIICS (CS peptides) for their effects on B16-F10 murine melanoma cell adhesion to the parent FN molecule, two nonadjacent CS peptides, designated CS1 and CS5, were found to inhibit melanoma adhesion to FN. The CS1 peptide and the CS5 peptide (containing the active sequence REDV) are located at the amino terminal and carboxyl-terminal ends of the alternatively spliced type III connecting segment, respectively. When amino-terminal cysteine derivatives of the CS peptides were chemically conjugated to IgG, both the CS1 and CS5 conjugates were found to promote melanoma cell spreading. One interesting aspect of this newly identified cell type-specific cell attachment region is that the 25-residue CS1 peptide is only 2-3-fold less active than intact FN. The biological activity of the CS5 peptide was similar to the RGDS peptide from the central cell-binding domain, i.e. several hundredfold less active than FN on a molar basis. Therefore, CS1 appears to be a major site for interaction of human FN with the melanoma cell surface. The receptor for CS1 has been isolated and identified recently.
as the $\alpha\beta$ integrin complex. Since the CS1 and CS5 sequences are each found in separate alternatively spliced regions of the IIICS, it is conceivable that the adhesion-promoting activity of FN may be under complex post-transcriptional regulation. Indeed, a recent report on the biosynthesis and gene splicing of fibronectin in epithelium suggests that alternative splicing may be used during wound healing as a mechanism to generate various forms of FN that are functionally more appropriate for the cell migration and proliferation associated with tissue repair [Pierschbacher and Rouslathi, 1982]. Furthermore, migratory assays of avian neural crest cells in vitro indicate that both the central cell-binding domain and the IIICS are required in association, each with functional specificity, to permit effective locomotion. These studies suggest the possibility of an instructive role for fibronectin in the control of adhesiveness.

A 33 kDa heparin binding fragment of fibronectin could promote tumor cell adhesion [McCarthy et al., 1986] as well as the RGD- or YIGSR- related peptides. CS1 and CS5 peptides which were present within the type III connecting segment domain (IIICS) of 33 kDa heparin F10 melanoma cell adhesion through an RGD-independent mechanism [Humphries et al., 1986; Humphries et al., 1987]. The minimal active sequence with in CS1 and CS5 were Leu-Asp-Val (LDV) [Komoriya et al., 1991] and Arg-Glu-Asp-Val (REDV), respectively.

### 2.3. Remedies for Tumor Treatment

#### 2.3.1. Obstacles for Curative Therapy of Cancer

The design of cancer chemotherapy has become increasingly sophisticated, yet there is no cancer treatment that is 100% effective against disseminated cancer. Resistance to treatment with anticancer drugs results from a variety of factors including individual variations in patients and somatic cell genetic differences in tumors, even those from the same tissue of origin. Frequently resistance is intrinsic to the cancer, but as therapy becomes more and more effective, acquired resistance has also become common. The most common reason for acquisition of resistance to a broad range of anticancer drugs is expression of one or more energy-dependent transporters that detect and eject anticancer drugs from cells, but other mechanisms of resistance including insensitivity to drug-induced apoptosis and induction of drug-detoxifying mechanisms probably play an important role in acquired anticancer drug resistance.
Studies on mechanisms of cancer drug resistance have yielded important information about how to circumvent this resistance to improve cancer chemotherapy and have implications for pharmacokinetics of many commonly used drugs. Here we have taken close look to some of them in the following lines.

**Construct and physiology of cancer** have tremendous effect on the effective delivery for the treatment of cancer. To reach to the cancer vasculature, leaky nature helps most for the small molecules. Again relation between the cancer cells, blood vessels, epithelium and basement membrane has impact on the effect treatment. The supporting stroma have critical role in the formation of new blood vessels and the growth of the cancer. The tumor possibly not grows beyond 1-2 mm in diameter without new blood vessels, which supplies nutrient to the cancerous cells. The changes occur at capillary levels which may rupture endothelium or basement membrane. Hence, ultimately the overall therapy or regimen will be depending on the construct or physiology of the cancer.

Variation in human's **physiology creates many problems** for treatment of cancer. Human tumors contain well perused, rapidly growing region as well as poorly perused, often necrotic regions. So the first obstacles to effective systemic treatments are the heterogeneity of the distribution of areas of growth within the tumor. The next barrier to appropriate delivery of cytotoxic agents is the transport of agents across the blood vessel wall in to the interstitium. In normal tissues, an intact endothelium acts as a selective barrier to all but the smallest molecules and ions. Larger molecules may penetrate by para or trans cellular pathways and in some cases by active transport. Barrier function in tumors is often inadequate due to compromised endothelial integrity. Because of this reduced integrity, access for drugs and macromolecules such as antibodies and liposomes can be increased. However, hydrodynamics and solute behavior influence the movement of such agents and the net effect of diffusive and convective forces may differ considerably from that predicated from observations on normal tissues.

**Problems at cellular level**

It came as something of a surprise that the major mechanism of multidrug resistance in cultured cancer cells was the expression of an energy-dependent drug efflux pump, known alternatively as P-glycoprotein (P-gp) or the multidrug transporter [Juliano and
Chapter 2.

Ling, 1976; Ueda et al., 1987]. This efflux pump, the product of the MDR1 gene in the human [Chen C-J et al., 1986] and the product of two different related genes, mdr1a and mdr1b in the mouse [Croop et al., 1989; Lothstein et al., 1989], was one of the first members described of a large family of ATP-dependent transporters known as the ATP-binding cassette (ABC) family [Higgins, 1992]. Every living organism has encoded within its genome many members of this family, and they appear to be involved not only in efflux of drugs but in moving nutrients and other biologically important molecules into, out of, and across plasma membranes and intracellular membranes in cells. P-gp is widely expressed in many human cancers, including cancers of the gastrointestinal (GI) tract (small and large intestine, liver cancer, and pancreatic cancer), cancers of the hematopoietic system (myeloma, lymphoma, leukemia), cancers of the genitourinary system (kidney, ovary, testicle), and childhood cancers (neuroblastoma, fibrosarcoma) [Goldstein et al., 1989]. The human gene most closely related to MDR1 is MDR2, a phosphatidylcholine transporter expressed in liver whose defect results in inability to form bile and progressive cirrhosis [De Vree JML et al., 1998]. P-gp can detect and bind a large variety of hydrophobic natural-product drugs as they enter the plasma membrane. These drugs include many of the commonly used natural product anticancer drugs such as doxorubicin and daunorubicin, vinblastine and vincristine, and taxol, as well as many commonly used pharmaceuticals ranging from antiarrhythmics and antihistamines to cholesterol-lowering statins [Bogman et al., 2001] and HIV protease inhibitors [Lee and Gottesman, 1998]. Binding of these drugs results in activation of one of the ATP-binding domains, and the hydrolysis of ATP causes a major change in the shape of P-gp, which results in release of the drug into the extracellular space [Ramachandra et al., 1998].

Various kinetic hurdles which leads ineffective cancer therapy includes poor absorption or rapid metabolism or excretion of a drug, resulting in low serum levels; poor tolerance to effects of a drug, especially in elderly patients, resulting in a need to reduce doses below optimal levels; inability to deliver a drug to the site of a tumor, as could occur with bulky tumors or with biological agents of high molecular weight and low tissue penetration such as monoclonal antibodies and immunotoxins (Pluen et al., 2001); and various alterations in the host-tumor environment that affect response of the tumor including local metabolism of a drug by non tumor cells, unusual features
of the tumor blood supply that may affect transit time of drugs within tumors and the way in which cells in a cancer interact with each other and with interstitial cells from the host (Green et al., 1999).

![Fig 2.05 Efflux mechanism of cell](image)

The Fig. 2.05 summarizes many of the ways in which cultured cancer cells have been shown to become resistant to cytotoxic anticancer drugs. The efflux pumps shown schematically at the plasma membrane include MDR1, MRP family members, and MXR (ABC G2), which is presumed to function as a dimer.

### 2.3.2. Constraint to conventional cancer therapy

Treatment of cancer is still ambiguous due to its limitations. Present therapies available to alleviate cancer are surgery, radiation and chemotherapy, which have its own limitations. The surgery is not possible in not to reach area. The radiation may burn natural cells of adjacent organ which have deleterious effect and the main demerit of chemotherapy is non specificity for example, do not differentiate between the normal and cancerous cells. These therapies are only effective if the cancer has been detected in early stages. Other therapies like sound, natural, but are not effective once the cancer becomes malignant. The available chemotherapeutic agents are very potent with narrow therapeutic indices, which will cause harm to normal cells and therefore the dosing of the chemotherapeutics are biggest challenge for the physicians.
2.3.3. Targeted drug delivery systems

Tumor targeting

Cells have on their surface specific molecules designed to regulate several processes such as differentiation and growth. These surface molecules can be overexpressed on tumor cells and are therefore referred to as tumor associated antigens. Tumors are also known to overexpress receptors, for example, growth hormones, vitamins and lipids. These overexpressed structures can be targeted and used for therapy by an antibody or a receptor ligand to which a toxic substance of a radionuclide has been coupled (Fig 2.06).

Antibodies, most frequently monoclonal antibodies, mAbs, are used to target tumor specific structures in several ways. In radioimmunotherapy, RIT, the radionuclide attached to the antibody is chosen to deliver local radiation energy in order to kill the targeted cells efficiently. The most frequently used radionuclides for therapy are $\beta$-emitters like $^{131}I$ and $^{90}Y$ while in radioimmunodiagnostic, RID the same targeting principle is applied but the radionuclides are chosen to emit X-ray and gamma suitable for external detection, for example $^{111}In$ and $^{99mTc}$. So far, successful therapy has been accomplished with haematopoetic tumors, such as non-Hodgkin's lymphoma. Antibodies targeting the tumor antigens CD-19, CD-20, CD-22 or CD-37 have been used with $^{131}I$ or $^{90}Y$ and have shown good specificity and therapeutic results. A humanized antibody, rituximab, directed towards CD-20 has shown good treatment effects (60% response) not just as a radiolabeled antibody, but also in it. A non-humanized version of this antibody, ibritumomab, has shown response rates of 80 % when labeled with $^{90}Y$ [Goldenberg, 2002; Postema et al., 2001]. Tumor cells can also be eradicated using antibodies conjugated to toxic substances, such as ricin, genistein and pseudomonas exotoxin A (ETA). Antibodies can be used for immunotherapy by themselves or conjugated to a super antigen to evoke a more
powerful immuno reaction towards the targeted tumor structure. The use of antibodies has been hampered by the fact that most mAbs are derived from mouse and can therefore evoke an immune response towards the injected murine antibody thereby disabling further injections. By changing the non-binding parts of an antibody to human parts, a humanized chimeric antibody is created, being much more tolerated for repeated dosing. In some cases when a smaller targeting agent is needed, only the binding part of the antibody, the Fab fragment can be used. The smallest parts of the antibody, the variable regions, so called single-chain fragments, ScFv, can also be used for targeting.

If the targeted structure in question has a natural ligand, then this ligand, or a derivative of it, can be used for targeting. For the overexpressed epidermal growth factor receptor, EGFR, the ligand EGF can be used for targeting. The vitamin folate receptor is often overexpressed on various types of tumors, such as ovarian, colorectal and endometrial carcinomas [Sudimack and Lee, 2000], and the folic acid or folate has been used for targeted delivery of both radionuclides and drug carriers [Sudimack and Lee, 2000]. Neuroendocrine tumors often overexpress the somatostatin receptor, and a somatostatin analogue, octreotide, has been used for both imaging and therapy of this kind of tumors [Carlsson et al., 2003]. Many types of tumors also overexpress receptors for low density lipoproteins, LDLs. This has awakened the interest for use of LDLs as delivery vehicles for chemotherapeutics. Experiments have been performed to load anthracyclins into LDL particles with promising results regarding stability and toxicity [a Masquelier et al., 2000; b Masquelier et al., 2000].

There are several ways to deliver the toxic agents with targeted therapy. The simplest way is to attach ligand to the surface of the carrier systems like liposomes or nanoparticles. The ligand can be antibody, peptide [RGD, YIGSR, EILDV], carbohydrate molecules [folate, mannose, lactin], proteins [transferring] ect. During initial studies it was found that the small particle size (> 200nm) will help to evade the RES and provide longer circulation and at the same time it will be internalize easily in the leaky capillaries of the tumor. However the problem of toxicity remains same due to its inefficacy to differentiate between the normal and pathogenic cells. In late 90’s research has been focused on the more specific delivery toward the tumor and treatment for the metastasis. The best suitable option for it is to prepare guided drug delivery which will specifically bind to the cancerous cells and make patient free from
the sufferings. In the present scenario the liposomes are proven to be good carrier for surface modification.

2.3.4. Liposomes
Liposomes are phospholipid bilayer spheres composed of lipophilic double membranes with an aqueous core. Liposomes have been proposed as drug delivery vehicles since the mid 1970’s [Gregoriadis G., 1974]. Hydrophilic drugs can be loaded in the aqueous core and lipophilic drugs in the double membrane. The early liposome in vivo experiments, using large (>200 nm), often multilamellar liposomes [Fidler et al., 1980], had problems with rapid removal from the blood stream by cells of the mononuclear phagocyte system MPS [Gabizon A. and Papahadjopoulos D. 1988]. To circumvent this problem, sterically stabilized liposomes were constructed and examined during the late 1980’s, where a polymeric coat was used to shield the liposomes from opsonization and recognition by the cells of the MPS [Gabizon A. and Papahadjopoulos D. 1988]. Two main formulations of stabilized liposomes were proposed: liposomes with monosialoganglioside, GM1 [Gabizon, A. and Papahadjopoulos, D.1988] and liposomes with polyethylene glycol, PEG [Klibanov et al., 1990; Allen et al.,1991]. Liposomes have been shown to accumulate in sites with increased capillary blood-flow and leaky vasculature, such as inflammations [Bakker-Woudenberg 1993; Boerman et al.,2001] and tumors [Forssen et al.,1992; Gabizon et al.,1992]. This tumor-homing effect is used for all commercially available liposome formulations today.

Some of the commercially available liposomal formulations are depicted in the table 2.03. This includes Myocet™ (Elan Pharmaceuticals) consists of doxorubicin enclosed in moderately sized liposomes (190 nm), DaunoXome® (Gilead Sciences) small liposomes (45 nm) loaded with daunorubicin are used. This is the case for the doxorubicin loaded liposome formulation known as Doxil®/Caelyx® (Alza Corporation). The liposomes are small (100 nm), rigid and coated with approximately 5 mol% PEG. Not only drugs for tumor therapy have been developed, AmBisome (Gilead Sciences) is a liposomal formulation of amphotericin B proven very effective against fungal infections. Amphotericin B forms an ionic complex with the phospholipids in the bilayer and is not, compared to the formulations with anthracyclins described above, loaded in the aqueous core. AmBisome was designed as very rigid, small, unilamellar liposomes (<100 nm) with long circulation times.
Table 2.03 Commercially available liposomal formulations

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Drug</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocet™</td>
<td>Elan</td>
<td>Doxorubicin</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>DaunoXome®</td>
<td>Gilead</td>
<td>Daunorubicin</td>
<td>Kaposi’s sarcoma (KS)</td>
</tr>
<tr>
<td>Doxil®/Caelyx®</td>
<td>Alza</td>
<td>Doxorubicin</td>
<td>KS, ovarian carcinoma</td>
</tr>
<tr>
<td>AmBisome</td>
<td>Gilead</td>
<td>Amphotericin B</td>
<td>Anti fungal</td>
</tr>
</tbody>
</table>

2.3.5. Tumor targeting liposomes
To increase the tumor-specificity of liposomes a targeting agent can be attached. There have been several strategies for attachment of tumor targeting agents, but the prevailing and most successful is to attach the targeting agent to the distal end of a PEG molecule on the outside of the liposome. It is already proved that the attachment of ligand to polymer increases the targeting ability and half life compared free ligands. There have been several conjugation-chemical approaches to achieve this [Nobs et al., 2004].

Several studies, both in vitro and in vivo, have been performed using targeted liposomes; so far none have performed clinical studies through. Among the most studied tumor targets with liposomes are the folate receptor, Human EGF receptor 2 (HER-2), CD-19, transfarir receptor and the anti-adhesion sequences like RGD and YIGSR.

Poly (ethylene glycol) (PEG) is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnical applications. In the modification of biological macromolecules, peptides and proteins are of extreme importance. Reasons for Pegylation (i.e. the covalent attachment of PEG) of peptides and proteins are numerous and include shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the reticuloendothelial system (RES), and preventing recognition and degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing the renal filtration and altering biodistribution. An important aspect of Pegylation is the incorporation of various PEG functional groups that are used to attach the PEG to the peptide or protein. When PEG is properly linked to a polypeptide, it modifies many of its features while the main biological functions, such as enzymatic activity or receptor recognition, maybe maintained and again
preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes.

**Table 2.04** Conjugation methods to couple ligands to liposomes

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Ligand</th>
<th>Anchor</th>
<th>Schematic presentation</th>
</tr>
</thead>
</table>
| Thioether | Fab' fragments SATA/SPDP modified ligand | MPB-PE, MP-PEG-PE, MMC-PEG-PE, MP-PE, PDP-PE, PDP-PEG-PE | ![Thioether Reaction Diagram](image)
| Disulfide | PDP/SATA modified ligand Fab' fragments | PDP-PE | ![Disulfide Reaction Diagram](image)
| Carboxyamide | Ligand - NH$_2$ | Ester NHS of fatty acids | ![Carboxyamide Reaction Diagram](image)
| Amide | Ligand - NH$_2$ | PE-PEG-COOH NGPE | ![Amide Reaction Diagram](image)
| Hydrazone | Oxidized ligand | Hz-PEG-PE | ![Hydrazone Reaction Diagram](image)

To couple PEG to a molecule (i.e. polypeptides, polysaccharides, polynucleotides and small organic molecules) it is necessary to activate the PEG by preparing a derivative of the PEG having a functional group at one or both termini. The functional is chosen based on the type of available reactive group on the molecule that will be coupled to the PEG. Various strategies for attachment of ligand to the surface of the liposomes were enumerated in the table 2.04.
The HER-2 has been studied as a target since early 1990's. Suzuki et al., (1995) studied doxorubicin loaded liposomes with antibodies targeting either the p185 residue or the p125 residue, and it was shown that targeting p185 was superior. All further studies have targeted this epitope on HER-2. Goren et al. (2000) showed that the uptake in cell culture was 16 times better for HER-2 targeted liposomes than non-targeted. Kirpotin et al., (1997) constructed immunoliposomes with Fab-fragments targeting HER-2, and they showed good binding and proven endocytosis in vitro. Park et al., (2002) have studied HER-2 targeting extensively and have shown therapeutic efficacy in several animal studies. They have tested liposomes with both Fab-fragments and single chain fragments, ScFv, against the p185 epitope, and both conjugates showed equal effect. Lopez de Menezes et al (2000) have targeted the tumor antigen CD-19 successfully on B-lymphoma cells both in vitro and in vivo. The in vivo studies in mice showed that doxorubicin in immunoliposomes targeting CD-19 gave much better results than free doxorubicin or doxorubicin in non-targeted liposomes. As a test of the specificity, liposomes with a non-idiotypic antibody were used with very limited uptake. Sarti et al. (2000) showed that transferrin liposomes interacted specifically with cultured cells and that they were internalized via receptor mediated endocytosis. Linuma et al. (2002) developed cisplatin loaded transferrin liposomes that proved to increase the cisplatin levels of disseminated tumor cells in ascites significantly. It was also shown by electron microscopy that gold labeled transferrin liposomes were located on the plasma membrane of cultured cells or in endosomes in the process of endocytosis.

Liposomes have been proposed as carrier for peptide targeted delivery to avoid demerits of the antibody and or protein targeted liposomes. Maximum work has been carried out on the RGD sequence of fibronectin, a component of ECM and play role in adhesion and other biological activities. Schraa et al., (2002) reported that chemically coupled monocyclic RGD peptides (cRGDfK) to an irrelevant human antibody resulting in a multivalent macromolecular peptide-protein conjugate developed for targeting therapeutic agents or immune effector cells to tumor vasculature. In another study same group studies RGDpep-HuMab conjugates bound to endothelial cells with more than thousand-fold increase in αvβ3/αvβ5 binding avidity. Furthermore, they have showed that RGDpep-HuMab conjugates became
internalized and degraded into primary endothelial cells in their study investigated that the RGD coupled to liposomes have effect in the experimental arthritis by means of the peptide have tendency to target αvβ3 integrins expressed on angiogenic vascular endothelial cells (VECs). They are able to bind VECs at sites of inflammation. Brain delivery also has been performed using RGD coated liposomes with ferulic acid [FA], the concentration of FA in brain was 6-fold higher than that of FA solution and 3-fold higher than that of uncoated liposomes [Qin et al., 2007]. The efforts were made to prepare surface modified liposomes for combretastatin A4 using RGD as targeting ligand for tumor vasculature by Nallamothu R et al., (2006). The findings were suggested that RGD modified liposomes showed significantly higher binding to their target cells than non targeted liposomes in the in vitro model system examined. This targeting effect may increase the anticancer activity of the drug and potentially improve its therapeutic benefits compared with non targeted liposomal or solution dosage forms.

Another antimaetastic sequence was used for targeting is YIGSR, a laminin sequence and expressed via either 67 kD laminin receptor or integrin receptor. In vivo stability of the peptides is enhanced by coupling the peptide with the polymer like PEG. The attempt were made by Maeda et al., they have studied PEG hybrids of YIGSR and other small laminin-related peptides and reports a facile synthesis of PEG hybrids of YIGSR (PEG–YIGSR, YIGSR–PEG, PEG–YIGSR–PEG) by the solid phase method [Maeda et al., 1998; Maeda et al., 2001]. Nishiyama et al., (2000) reported efficient conjugation of YIGSR with the chitosan, this conjugation showed higher antimetastatic activity. Attachment of YIGSR with the PVP, a hydrophilic polymer increases the half life and antimetastatic activity. PVP<sub>5000</sub> was proved to be the best among the other polymer used like PEG of different molecular weight [Mu Yu, 1999]. Witkowska et al., (2004) and coworkers synthesized eleven YIGSR analogues by the solid phase method, and their biological activity has been studied in vitro by a cell adhesion assay: all of them inhibited the adhesion of LLC tumor cells to laminin. The analogues were found to be more resistant to enzymatic degradation in human serum than YIGSR-NH<sub>2</sub> itself. Analogue DatIGSHar-NH2 was selected for an experimental pulmonary metastasis assay in vivo: it had higher antimetastatic activity than YIGSR-NH<sub>2</sub>. 

47
EILDV the sequence from the fibronectin and again expressed via integrin family have potent anti-adhesive and therefore antimetastatic activity on the metastatic cells. Very few reports are available on the EILDV activity. An amino acid type poly (ethylene glycol) (aaPEG) was prepared and its application to a drug carrier was examined. The peptides, Arg-Gly-Asp (RGD) and Glu-Ile-Leu-Asp-Val (EILDV) which were reported as ac- fragments of Fibronectin (a cell adhesion protein), were conjugated with aaPEG (molecular weight, 10,000). The synthesis was carried out using solid phase and solution phase method. It is concluded that the conjugation with the PEG dose not modify the antiadhesive activity of the peptide, in addition increase the half life and therefore the activity [Maeda et al., 1997]. Yamamoto and workers examined the activity of three synthetic peptides derived from type III connecting segment domain [IIICS]. The sequences include EILDV [Glu-Ile-Leu-Asp-Val], EILDVPST [Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr], REDV [Arg-Glu-Asp-Val] and laminin related peptide YIGSR [Tyr-Ile-Gly-Ser-Arg]. The results reveal that each peptide inhibited experimental metastasis induced using B16F10 melanoma cells. The pegylation have better activity and EILDV had strongest inhibitory effect among all peptides [Yamamoto et al., 1994]. In this work, solid phase synthesis and solution phase method was used. The sequence synthesized were from the III connecting segment domain of fibronectin i.e. HEILDV-NH₂, HEILDVPST- NH₂ and REDV-NH₂. The sequence EILDV and REDV exhibited inhibitory effect on the experimental metastasis and EILDV emerge as most potent inhibitor. The EILDV-PEG-REDV sequence dramatically increases the inhibitory effect on the metastasis [Kawasaki et al., 1996].

Lopez et al (2004) represent the liposomal system encapsulating doxorubicin in liposomes coupled to YIGSR peptide. The observation was very challenging as this drug delivery enjoys the dual benefit of specificity for tumor and lower toxicity. The other work has been proposed by Dubey et al., (2004) on the cyclic RGD peptide. They encapsulate 5- fluorouracil in to the stearically stabilized liposomes and Spontaneous lung metastasis and angiogenesis assays show that RGD peptide anchored liposomes are significantly effective in the prevention of lung metastasis and angiogenesis compared to free 5-FU, stealth liposomes and RAD-SL, a control peptide liposomal attaché to liposomes.
2.4. **IN VITRO CELL LINE STUDIES**

Cells in culture or in vitro are a useful model for studying the activity of cells in the whole organism or in vivo. Ten years ago or so cell culture techniques were considered somewhat esoteric. Today because of our better understanding of cell nutrition, metabolism and general growth environment it has become a fairly routine procedure. The cell line studies are useful to evaluate therapeutic potency of the active materials on the specific cells. The main advantage of in vitro cell line is reduces the time required and it is cost effective in comparison to animal study. Various reports on cell line studies were already existed in the literature (REF). The basic study includes cytotoxicity assay, colony formation assay, cell motility assay (wound assay), cytopathic study, adhesion study, cell cycle analysis and cellular uptake studies. Zymography study, western blotting, cell internalization assay can be performed using cell lines.

Canfeza S. et al (2002) evaluated the cytotoxic and apoptotic effects of a new candidate cytotoxic compound, As2O3 (arsenic), alone and in combination with taxanes on breast cancer cell line MCF-7 as a model system. They concluded that As2O3 is cytotoxic on breast cancer cells. Paclitaxel and docetaxel showed promising effects in combination with As2O3, and clinical trials with these combinations are warranted. The B16F10 cells expressing high levels of CD44 avidly bind and internalize HAL in a temperature dependent manner, whereas cells expressing low levels of CD44 do not. Eliaz and Szoka, Jr (2001) in their important finding reported that DOX (doxorubicin) encapsulated in HAL is more potent than the free DOX in both transient and continuous exposure conditions for periods up to 24 h. Thus, the HALs may provide an effective vehicle for delivering chemotherapeutic agents into CD44-expressing tumors in animals. This findings supports the effective targeting to the over expressed receptor present on the cancerous cells.

Triptolide inhibits the growth and metastasis of solid tumors (Yang et al. 2003). Triptolide blocks growth of cancer cell line of distinct origin and different p53 status (B16 mouse melanoma, MDA-435 human breast cancer, TSU bladder cancers, and MGC80-3 gastric cancer). Again the tumor regression potential triptolide were comparable with other conventional chemotherapeutic drugs, such as Adriamycin,
mitomycin, and cisplatin. They also concluded that triptolide influences the expression of key molecules that regulate apoptosis and cell cycle progression. Cell traversing activity assay (wound assay) was used to find out migration ability of the cells. The other two are cell-invasive motilities: cell infection and cell-traversal motility (Mota et al. 2001; Kappe et al. 2003). Cell-infection motility is accompanied by vacuole formation and is followed by parasite development into exo-erythrocytic forms (EEFs). Cell-traversal motility, on the other hand, involves plasma-membrane disruption and is followed by migration through the cytoplasm and eventual escape from the cell. Recently, Mota and Rodriguez (2002) revealed that this type of cell-invasion motility can be identified by conventional cell-wound assay. According to the observation that passage through some hepatocytes by this motility precedes hepatocyte infection, they proposed the hypothesis that this motility is necessary for sporozoites to be activated for hepatocyte infection (Mota and Rodriguezet, 2002). Ishino et al (2004) used to cell lines, HepG2 or HeLa to show the cell passage activity required for the malarial parasite to cross the cell layer.

Qian et al (2007) studies the role of endogenous PRL-3 in the whole metastatic process of B16-BL6 cells from footpad to draining lymph node in C57BL/6J mice. In addition they have also evaluated the clinical ability of PRL-3 siRNA to prolong the survival time of mice in the spontaneous metastasis model. The cell cycle analysis was also performed on same cell lines. In the same study they have performed the adhesion and invasion assay and find that the SiRNA inhibits the invasion and adhesion of the B16-BL6.

2.5 IN VIVO STUDIES

Tumor has remarkable effect on the pharmacological parameters (both kinetic and dynamic). The kinetic and dynamic study in suitable animal model gave idea about the absorption, distribution and excretion of the drug and efficacy of the drug delivery. The rout of administration also modified the kinetic parameters. Tissue distribution data pertaining to the drug provide information about accumulation of the drug in the specific organ, a good tool for determination of toxicity and efficacy of drug delivery.
Beer et al., (2005) performed a study for detailed characterization of the biodistribution and pharmacokinetics of 18F-Galacto-RGD in cancer patients. They had chosen patients with melanomas and musculoskeletal sarcomas, because the role of αvβ3 in angiogenesis and metastatic potential has already been described for these tumors. Moreover, phase I and phase II studies with humanized anti-αvβ3 antibodies have already been performed in patients with soft-tissue sarcomas. The positron emission topography was used to evaluate the distribution and kinetic parameters in the cancer patients. They concluded that 18F-Galacto-RGD demonstrates a highly favorable biodistribution in humans with specific receptor binding, by allowing visualization of αvβ3 expressions in tumors with high contrast. Consequently, this tracer offers a new strategy for noninvasive monitoring of molecular processes and may supply helpful information for planning and controlling of therapeutic approaches targeting the αvβ3 integrin. The evidence for the mechanisms underlying increase antitumor efficacy dose not involve enhanced accumulation of anti-HER2 immunoliposomes in tumor tissue due to antigen binding. Rather, both liposomes and immunoliposomes localized at high levels in tumor tissue, but revealed a marked difference in pharmacodynamic with respect to tumor cells in vivo: immunoliposomes, but not liposomes, mediated intracellular drug delivery to HER2-overexpressing cancer cells in animal models (Kirpotin et al., 2006). Kaul and Amiji (2004) developed a safe and effective systemically-administered biodegradable nanoparticles delivery system for solid tumors and examined the comparative biodistribution profiles of gelatin and poly(ethylene glycol) (PEG)-modified (PEGylated) gelatin nanoparticles in subcutaneous Lewis lung carcinoma (LLC)-bearing female C57BL/6J mice. They found that PEGylated gelatin nanoparticles do possess long circulating properties and can preferentially distribute in the tumor mass after systemic delivery.

Various reports are available in the literature on the metastasis assay in the suitable animal model. Dora et al., (2006) evaluate the pulmonary antimetastatic activity and the systemic toxicity of camptothecin-loaded microspheres. The ability of camptothecin to inhibit the lung metastasis was verified using an experimental mouse model intravenously injected with metastatic B16-F10 melanoma cells. The microspheres and the free drug were given intraperitoneally at a dose of 7 mg/kg at
intervals of three or five days for 24 days. The systemic toxicity of camptothecin was evaluated by weight measurements, survival and hemograms of the animals. From the study it was concludes that camptothecin-loaded microspheres demonstrated similar therapeutic efficacy when compared to those of the free drug, but the toxicity was significantly reduced.


**REFERENCE**


Colucci S., Giannelli G., Grano M., Faccio R., Quaranta V., and Zallone A.Z., Human osteoclast-like cells selectively recognize laminin isoforms, an event that induces migration and activates 


Chapter 2


Yamamura K., Kibbey M.C., Kleinman H.K., Melanoma cells selected for adhesion to laminin peptides have different malignant properties, Cancer Res. 53 (2); 423-428; 1993.


