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**MORPHOLOGIC ASPECTS OF THE BASIC CELL
BIOLOGY OF CANCER**

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Morphologic methods offer unique opportunities to study some of the characteristics of malignant and normal cells. One of the promising areas of cancer therapy is the use of targeted toxins, such as immunotoxins, that can selectively destroy tumor cells. We have used morphologic approaches to the screening and evaluation of monoclonal antibodies for use as part of immunotoxins directed against human ovarian cancers. Screening methods that utilize immunofluorescence and immunoperoxidase methods have allowed the rapid isolation of hybridomas that react selectively with tumor cells in tissue culture and in solid tumors, while showing only limited reactivity with important normal tissues. Another area of cancer biology in which morphologic methods have proved useful is the study of multidrug resistance of human tumors. The efflux activity seen in single multidrug resistant cells can be demonstrated morphologically using fluorescent chemotherapeutic drugs, such as daunomycin. The protein responsible for this efflux pump activity, P170, has been localized morphologically to the plasma membrane in individual cultured cells, as well as located using immunohistochemistry in normal liver, kidney, GI tract, adrenal and cerebral capillaries. These locations enhance understanding of the role of this protein in the excretion of toxic hydrophobic compounds, both in chemotherapy and in normal organ physiology. These methods also allow simple and rapid screening techniques to evaluate other drugs that might be able to reverse the multidrug resistance phenotype and render human tumors more sensitive to chemotherapy.

Microscopy has long been a tool of basic biological science. Advances in the understanding of biochemical events in intact cells have always required the precise knowledge of the location of the chemical constituents involved. Thus, histochemistry and cytochemistry have a pivotal role to play in future advances in cell biology. One of the areas that has recently benefited from cytochemistry is the understanding of some of the cellular and molecular biological events related directly to cancer therapy. In this paper, I will describe two subjects of this type related to cancer therapy and diagnosis: the development of immunotoxins and the study of multidrug resistance.

MATERIALS AND METHODS

The methods used for immunofluorescence microscopy have been previously reviewed (7). The immunizations using intact tumor cells, isolation of hybridomas, and preparation of immunotoxins have been previously presented (6). Methods for the characterization of the efflux of daunomycin and the evaluation of drug that reverse multidrug resistance have been published elsewhere (5). The characteristics of the multidrug resistance phenotype and the isolation of the MDR1 gene has recently have reviewed (1). Methods for immunolocalization of P170 using immunofluorescence and immunoelectron microscopy (8) and the methods for immunoperoxidase localization of P170 in tissues (4) have also been previously published.

RESULTS AND DISCUSSION

Isolation and Characterization of Antibodies for Use in Immunotoxins

Advanced metastatic cancer is often characterized by the presence of tumor cells in many normal tissues in locations that are either not amenable to surgery, or contain cells that are relatively slow-growing. Such tumors are, thus, not sensitive to treatment methods such as radiotherapy or drug chemotherapy that depend on rapid growth of cells for toxic selectivity. Therefore, other systemic approaches to treatment that do not depend on rapid growth of tumor cells are needed. One method of selective cell killing that would allow destruction of tumor cells without damage to normal cells depends on targeting a toxic agent to tumor cells using a selective epitope on the cell surface. Such an epitope might be an antigenic epitope, or it might be a receptor that would selectively bind a ligand coupled to toxic materials. The toxic material might be a bacterial or plant toxin, or it might contain radionuclides or chemotherapeutic toxic drugs. Whatever the mechanism of cell killing, a key to success with such therapy is the selectivity of the targeting mechanism.

A common method of achieving targeting is the use of antibodies that react with tumor-specific epitopes on the surface of tumor cells. The advent of hybridoma technology allowed the generation of large amounts of pure antibodies reactive with epitopes that are present in amounts too low to be isolated and purified. The key element if such antibodies are to be used therapeutically is to select antibodies that react to epitopes that are as close to being completely tumor-specific as possible. In spite of many years of searching for such antibodies, very few monoclonals have fully met the test of selective reactivity to tumor cells with no reactivity to normal tissues. Much of the problem in isolating such antibodies resides in the screening methods and in the

histochemical methods used to evaluate specificity. The full understanding of the use of these methods will allow much improved antibody selection. While biochemical assays of tumor reactivity of such monoclonals are useful, morphologic methods offer the potential of evaluating the homogeneity of reactivity of antibodies to cells in intact solid tumors, as well as their reactivity to minor subsets of cells in normal tissues, some of which may be physiologically vital. Thus, our studies have been directed at the development of morphologic methods that would allow precise evaluation and isolation of tumor-specific antibodies.

Immunotoxins are the product of conjugating selective surface-reactive monoclonal antibodies with potent protein toxins, such as *Pseudomonas* exotoxin (reviewed in 3). A model of the entry of such an immunotoxin is shown in Fig. 1. It seems clear from most studies that endocytosis is a necessary step in the cell-killing

FIG. 1. A diagrammatic summary of the morphologic pathway of immunotoxin delivery to the cytoplasm through endocytosis. The intracellular site at which toxin escapes into the cytosol is unclear but, for *Pseudomonas* exotoxin, translocation probably occurs in the receptosome.

FIG. 2. The morphologic appearance of human ascites ovarian tumor cells using phase contrast microscopy. (Original magnification = $\times 200$)

mediated by such immunotoxins. After selective targeting to a surface epitope by the monoclonal antibody, the immunotoxin is internalized and the toxic component of the protein toxin is somehow translocated across an intracellular membrane barrier into the cytosol, where the toxin exerts its killing activity. The catalytic toxic activities of most large protein toxins used for this purpose are mediated by direct enzymatic actions on cytosolic components of the protein synthetic machinery. Thus, cells that bind the antibody-toxin complex are killed, and cells that do not interact with the antibody are not killed.

The interaction of the immunotoxin with normal cells, such as hepatocytes, is an important factor in determining the utility of such conjugates. Some cell types have inherent binding activity for the toxin itself. In the case of *Pseudomonas* toxin, human

FIG. 3. Immunofluorescence reactivity of co-cultured human ovarian carcinoma cells and normal human fibroblasts. a. Represents the paired phase contrast and immunofluorescence images of cells incubated with anti-human transferrin receptor. The ovarian tumor cells appear as flat ovoid cells, whereas the normal fibroblasts (marked with a target symbol) are elongated. Note that both cell types show binding of this antibody as detected by immunofluorescence in the right-hand panel. b. Shows the same type of experiment using a monoclonal antibody that is tumor cell-specific. The normal fibroblasts (targets) show no reactivity with the antibody, whereas the tumor cells are all reactive (right panel). (Original magnification = $\times 300$)

FIG. 4. Accumulation of daunomycin in nuclei of multidrug-resistant and sensitive cultured human carcinoma cells. KB cells (a, c), a human carcinoma cell line and KB-C4 cells (b, d), a multidrug-resistant derivative cell line of KB, were incubated with $10 \mu\text{M}$ daunomycin for 5 min at 37°C , and then fixed using formaldehyde. The inherently fluorescent daunomycin accumulated in nuclei in these intact cultured cells was visualized using rhodamine epifluorescence optics. (a) shows that normal drug-sensitive cells accumulate large amounts of daunomycin in their nuclei in this short incubation, whereas in (b) the multidrug-resistant derivative cell line (KB-C4) shows very little nuclear daunomycin accumulation in a similar incubation. Note in panel (a) (inset) that the nuclear envelope plays no role in this accumulation, since mitotic chromosomes show the same accumulation of daunomycin as nuclear DNA. When these two cell types were exposed to daunomycin in the presence of $10 \mu\text{M}$ verapamil (c, d), a drug known to reverse the multidrug resistance phenotype in long-term cell survival assays, the resistant cells (d) show the same accumulation of daunomycin as normal cells (c). This indicates that this daunomycin assay reflects similar changes in properties of the multidrug resistance phenotype as seen for cell killing assays. (Original magnification = $\times 600$)

cells appear to bind less toxin than rodent cells, and are consequently less susceptible to cell killing by the toxin alone. *Diphtheria* toxin, on the other hand, has the reverse species selectivity, as is well known for the extreme sensitivity of human cells to *Diphtheria* toxin. Thus, the elimination of this "non-specific" binding of normal cells by the toxin alone, and of the toxin-antibody complex, is an important step in the development of a truly tumor-specific immunotoxin of therapeutic benefit. The lack of reactivity of some mouse antibodies with human F_c receptors, for example, makes

Fig. 5. A diagrammatic summary of the entry, accumulation and efflux of daunomycin in multidrug-resistant cells. The structure labeled "efflux pump" represents the role of the multidrug transporter P170.

such mouse antibodies useful for human therapy. While many problems remain in the total elimination of non-specific binding of immunotoxins, the selection of antibodies with the greatest tumor-selectivity is still of paramount importance.

Our work has concentrated on the generation of antibodies that react with the surface of human ovarian carcinoma cells. Ovarian carcinomas frequently present as large ascites tumors. An example of the appearance of such ascites cells is shown in Fig. 2. In evaluating the potential reactivity of hybridoma antibodies to such tumor cells, we have employed immunofluorescence screening methods on living cultured cells. Co-cultures of ovarian tumor cells and normal human fibroblasts allow the early detection of hybridomas of interest in the primary screening step. An example of the immunofluorescence appearance of such a screening step is shown in Fig. 3. Antibodies that react with both normal and tumor cells are discarded, while antibodies that selectively react homogeneously with cultured tumor cells are saved for further screening. Secondary screening is performed using peroxidase immunohistochemistry on cryostat sections, in which reactivity with intact tumors and with important normal tissue such as liver and kidney are evaluated.

One antibody isolated using these methods is OVB3, which reacts uniformly with almost all ovarian adenocarcinomas, yet reacts with very few important normal tissues (6). Immunotoxins prepared using this antibody have proven to be effective both in tissue culture and in animal models of human ovarian cancer (6). Such an immunotoxin is currently undergoing clinical trials in human ovarian cancer patients.

The future isolation of other hybridomas that are tumor-specific will continue to rely heavily on morphologic and histochemical evaluations of specificity. Other potential advances include the design of modified toxins using recombinant DNA methods that will hopefully prove less toxic for non-specific sites, yet retain high levels of toxicity when targeted by a monoclonal antibody. Therapy with such agents may eventually entail a large panel of different antibodies and toxins, antibody-radionuclide conjugates, and antibody-drug conjugates that can be selectively chosen to match the epitope and sensitivity characteristics of each individual patient. The goal of these approaches is to achieve specific tumor destruction with a broad therapeutic window, where non-specific toxicity is kept to a minimum and elimination of the very last

FIG. 6. Immunoperoxidase localization of the multidrug transporter (P170) in human liver and adrenal using monoclonal antibody MRK16. Cryostat sections of human liver and adrenal were incubated with monoclonal antibody MRK16 that reacts with the endogenous form of the multidrug transporter in normal human tissues. Localization can be seen along the biliary face of hepatocytes in liver (a, arrowhead) and on the surface of cells in the adrenal cortex (b, arrowhead). (Bars = 15 μ m)

tumor cell is possible. Tumor-specific monoclonal antibodies also have potential in tumor imaging and pathologic diagnosis. A great deal of work remains to achieve these goals, but histochemical methods will clearly play a major role in this endeavor.

Morphologic Contributions to the Study of Multidrug Resistance

One of the major problems in the chemotherapy of human cancer is the phenomenon of multidrug resistance (reviewed in 1). Some tumors initially present with this multidrug resistance phenotype, while others develop these properties as a consequence of relapse following an initial remission after chemotherapy. The multidrug resistance phenotype is characterized by resistance of tumor cells to killing by many different chemotherapeutic drugs of unrelated chemical and biological properties. Such drugs include commonly used cancer chemotherapy agents such as anthracyclines (daunomycin), Vica alkaloids (vinblastine) and actinomycin D. Multidrug-resistant cells may be highly resistant to drugs to which they were never exposed. Many cell types, both animal and human, which are exposed to increasing levels of these drugs in tissue culture and develop multidrug resistance, spontaneously employ the same mechanism to evade drug toxicity. This mechanism has recently been characterized as a rapid efflux of drugs out of the cytoplasm of cells (1, 5). A direct demonstration of this efflux activity is provided by morphologic experiments that visualize the accumulation of the inherently fluorescent drug daunomycin (5). Fig. 4 shows such an experiment using paired human tumor cell lines. The parental

FIG. 7. Localization of the multidrug transporter in human kidney. Cryostat sections of human kidney processed as described in Fig. 6 show localization of the multidrug transporter on the apical surface of epithelial cells in proximal tubule (arrow). (Original magnification = $\times 200$)

cell line (KB) is highly drug-sensitive, while the derivative multidrug resistant cell line (KB-C4) is highly drug-resistant. When these cells are exposed for a few minutes to daunomycin in tissue culture, the sensitive cell line accumulates the drug in the nucleus of each cell, whereas the resistant cell line shows very little drug accumulation. Other agents that have been previously shown to reverse this drug-resistance phenotype, such as verapamil, reverse the properties of the resistant cell to those of the sensitive cell, and these cells then show accumulation of daunomycin in their nuclei. This cell-by-cell expression of the multidrug resistance phenotype in a short time allowed the careful evaluation of the reasons for the lack of accumulation of daunomycin in these resistant cells. Fig. 5 shows a model of the mechanism that was derived from these studies. The entry of drug into the cytoplasm appears to be the same in both cell types, and is an energy-independent passive diffusion across the plasma membrane. In the sensitive cells, the drug then is accessible to the nucleus, where it binds weakly to nuclear DNA. This is the fluorescence image that is shown in Fig. 4a. However, in the resistant cells, the drugs rapidly effluxed out of the cell by an energy-dependent efflux pump in the plasma membrane.

The efflux pump activity has now been linked to the expression of a single protein in the plasma membrane of such cells. This protein, termed "P-glycoprotein",

FIG. 8. A diagrammatic summary of the transport mediated by the multidrug transporter in normal human abdominal organs. The locations of the multidrug transporter in liver, GI tract, and kidney all contribute to excretion of chemotherapeutic drugs and other low molecular weight toxic compounds. The role of the transporter in the adrenal gland is not yet clear.

"P170", or "the multidrug transporter", has been identified in many different multidrug-resistant cell types (1). The corresponding human gene, *MDR1*, for this protein has been isolated, cloned and sequenced (1). The transporter is a trans-membrane protein of 170,000 MW which has multiple membrane-spanning domains and nucleotide binding sites identifiable in its primary sequence (1). Monoclonal antibodies to the externally exposed portions of this protein have been isolated. We have used one of these, MRK16 (4, 8), to localize this protein in the plasma membrane of multidrug-resistant cells in tissue culture, as well as to examine the distribution of such a protein in normal tissues (4). Fig. 6 shows immunoperoxidase localization of the multidrug transporter on the bile canalicular face of hepatocytes and on the surface of cells in the adrenal cortex. Another major location of this protein is the apical surface of the epithelial cells in the proximal tubules of the kidney (Fig. 7). Other sites of localization of the multidrug transporter include the surface of the epithelia of the middle and lower GI tract. Some of these locations and their role in excretion of drugs are summarized in the drawing shown in Fig. 8. Another location of this transporter is in capillaries of the central nervous system, where this protein may form an efflux pump at the blood-brain barrier. The primary sequence of P170 contains information that allows the protein to be expressed in a polarized fashion, as shown by its selective apical expression in MDCK cells in tissue culture that have been infected with a virus carrying the human *MDR1* gene (2). Thus, this protein represents a highly conserved efflux pump that is expressed selectively and precisely in important normal tissues. The reason for its expression may relate to the need to excrete toxic low molecular weight materials that humans and other mammals encounter in their environment.

The morphologic results in the characterization of single cell drug efflux and the location of the multidrug transporter have added important understanding to the role of this protein in both multidrug-resistant human tumors and in normal cell physiology.

CONCLUSIONS

Many of the results presented in this paper would not have been as evident using non-morphologic techniques. These areas of research provide important examples of the continuing importance of histochemical and cytochemical methods in both basic cell biology and in experiments of direct clinical significance. The understanding of biological phenomena in terms of biochemistry and molecular biology will always require a careful evaluation of the location of the individual components of each mechanism in a morphologic setting. It is, thus, the responsibility of histochemistry to establish those methods and approaches that will yield the most direct and accurate data. With this goal in mind, the future of histochemistry and cytochemistry remains bright and in the forefront of biological science.

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