ABSTRACT

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Integrins are heterodimeric transmembrane glycoproteins consisting of α and β subunits. These adhesive receptors interact with extracellular matrix proteins, which not only influence cell survival and proliferation, but also adhesiveness and migratory process. Progression of cancer is associated with a variety of changes in cell growth and proliferation, including changes in integrin expression, rearrangement and redistribution on cell surface. Various studies have discovered that integrins influence migration, differentiation, and apoptosis, processes pivotal in carcinogenesis. Therefore, determination of the expression patterns of integrins in various cancers is of primary interest. Esophageal cancer is a disease with a low survival rate. This cancer is widely prevalent in Japan, northern Iran, India, Thailand, South Africa and United States. The main causative agents of this cancer are believed to be alcohol consumption and smoking. The main objective of this study was to determine the expression of α3β1 and α6β4 integrins on rat esophageal tumorigenic (RE-B2T, RE-C1T) and non-tumorigenic (RE-79, RE-149, RE-282) cell lines. Normal rat kidney, trachea, and fibroblast cell lines were used as control. Monoclonal antibody 5A produced against α3β1 integrin and anti-α6β4 monoclonal antibody was used in this study. Expression of these two integrin molecules was determined at antigen and protein levels by ELISA and at mRNA level by RT-PCR. The ELISA results showed that these cell lines express both types of integrins differentially. However, the expression of these integrin molecules was higher on non-tumorigenic cell lines than tumorigenic ones. The expression of the β1 integrin appears to be higher on non-tumorigenic cell line than the tumorigenic one at the mRNA level as determined by RT-PCR. It was concluded that in in vitro culture system, rat esophageal cells express α3β1 and α6β4 integrin molecules differentially. It appears that the expression of these two molecules is down-regulated in the invasive cell line, RE-B2T,
compared to other non-tumorigenic or normal cell. This reduction in the expression may enhance tumor cell motility and contributes to the metastatic spread of tumor \textit{in vivo}
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INTRODUCTION

Integrins are a class of cell surface adhesion receptors found in many animal species, ranging from sponges to mammals. There are no homologs of this molecule in prokaryotes, plants or fungi (Whittaker and Hynes, 2002). As diploblastic organisms evolved, the two cell layers may have evolved separate integrins to mediate their asymmetric interactions with the basal lamina. Representative of these two primordial integrins are detected in all higher metazoan phyla (see review: Hynes, 2002). These receptors link the extracellular matrix to the cytoskeleton and cooperate with growth factor receptors to promote cell survival, cell cycle progression and cell migration (Giancotti and Rouslahti, 1999). Primarily integrins mediate the interactions between the cells and extracellular matrix through the formation of adhesive structure called focal adhesion (Giancotti and Rouslahti, 1999).

STRUCTURE OF INTEGRINS

The integrin molecules consist of α and β glycoprotein subunits. The subunits are held by non-covalent bonds. Each integrin subunit is composed of a large extracellular domain, a single transmembrane portion and a short cytoplasmic component (Giancotti, 2003). The N-terminal domains of the α and β subunits associate to form the integrin headpiece, which contains the ligand binding site. The C-terminal segments traverse the plasma membrane and mediate interaction with the cytoskeleton and with signaling protein (Giancotti, 2003). The cytoplasmic tails are generally small, containing approximately 30-50 amino acids. The N-terminal domain of each integrin α-subunit contains a 200 amino acids long inserted sequence, known as I domain (see review: van der Flier and Sonnerberg, 2001). The structural analysis of integrin I domains has revealed the presence of a Mg\(^{2+}\) dependent adhesion site which is required for ligand binding.
(Kanazashi et al., 1997; Plow et al., 2000). This domain dominates the ligand binding functions of integrins.

**INTEGRIN FAMILY AND LIGAND BINDING**

There are at least 18 distinct α and 8 β subunits of integrin, which can associate in various combinations to form 24 distinct integrin molecules (Hynes, 2002). Figure 1 depicts the various possible combinations of the two integrin subunits. These molecules have the ability to recognize multiple ligands. The ligand binding specificity of integrins is dependent upon the combinations of α- and β- subunits. Furthermore, several bacterial pathogens such as *Borrelia burgdorferi*, *Bordetella pertussis*, and *Yersinia species* as well as viruses such as adenovirus, coxsackievirus, echovirus, foot-and-mouth disease virus, HIV (Tat protein), hantavirus, and papillomavirus use integrins as their receptors (see review: Kerr, 1999). Some of these microorganisms utilize integrin to gain entry into cells (see review: van der Flier and Sonnerberg, 2001). There are direct and multiple linkages between integrins and host defense system (see review: van der Flier and Sonnerberg, 2001). The preference of any integrin for its ligands is determined by relative affinity, availability of the ligands within a specific microenvironment, and the conformational state of the ligand (Plow et al., 2000).
Figure 1: Various integrin receptors (Hynes, 2002).

**INTEGRIN ACTIVATION AND SIGNALLING**

Affinity of an integrin molecule to its ligands is dependent upon the cell in which it is expressed (Diamond and Springer, 1994; Hughes and Pfaf, 1998; Dedhar, 1999). Many integrins require activation prior to binding with the ligands, and this property plays an important role in the biological functions of integrin. αIIβ3, a major platelet integrin, which is also known as GPIIb/IIIa, requires activation by agonists (e.g. thrombin, epinephrine) for binding to its ligand. If this activation would not occur, the platelets would bind to their major ligand, fibrinogen, and would cause platelet-fibrinogen aggregation. (see review: Hynes, 2002). Integrin clustering enhances the binding avidity between integrin and its ligands. Both polyvalent binding capacities with ligands and interactions with cytoskeleton cause clustering of integrin, which in turn, increases the extracellular matrix binding (Giancotti, 2003).
**ALTERNATIVE SPlicing AND LigAND BINDING**

Various subunits of integrin undergo alternative splicing which cause variations in the extracellular and cytoplasmic sequences in both vertebrates and invertebrates (see review: van der Flier and Sonnerberg, 2001). The binding ability of integrin to its ligands is determined by the combined activity of the two domains of $\alpha$ subunits and the conserved region present in all $\beta$ subunits (Melker and Sonnerberg, 1999). The splicing of the $\alpha$ subunit of the integrin is tissue-specific and developmentally regulated (see review: van der Flier and Sonnerberg, 2001).

![Integrin Diagram](image)

**Figure 2:** Diagram represents the regions of an integrin $\alpha$ molecule where alternative sequences have been found (Melker and Sonnerberg, 1999).

**INTEGRINS IN CANCER**

The metastatic behavior and invasiveness of poorly differentiated cancers is characterized by up- or down regulation of integrins (Clezardin, 1998). These variations in the expression play important roles in tumor cell proliferation, migration and invasion. Transformed cells are characterized by their anchorage-independent growth which reflects tumor cells ability to metastasize in vivo. It has been suggested that constitutive activation of integrin signal transducers such as FAK, Rho and ILK could regulate the anchorage-independent growth of tumor cells (Giancotti and Rouslahti, 1999). Migration of cells is also dependent upon integrins, through repeated activations and deactivations of these molecules (Clezardin, 1998). Members of Rho subfamily of proteins and small GTP binding proteins are thought to be involved in the integrin signaling and subsequent cell migration.
\(\alpha_3\beta_1\) INTEGRIN

\(\alpha_3\beta_1\) integrin which is also known as very late antigen 3 (VLA3), is a promiscuous receptor and it can bind with collagen, laminin, fibronectin and kalinin/epiligrin (Plow et al. 2000). Epithelial cells have higher expression of this integrin molecule (Iyer and Pumiglia, 2005). Knockout mice experiments have shown the importance of \(\alpha_3\beta_1\) integrin, in maintaining the integrity of the basement membrane during embryonic development of epidermis and other tissues (DiPersio et al., 1997; Kreidberg et al., 1996). This molecule is expressed by most tumor cells. In some reports, higher expression of \(\alpha_3\beta_1\) is correlated with increased metastasis (Morini et al., 2000). Reduced metastasis by lung carcinoma cell lines have also been observed when the cells were treated with anti-\(\beta_1\) integrin antibody (Yoshimasu et al., 2004). \(\alpha_3\beta_1\) integrin interacts with urokinase receptor and tetraspanin, both of which play a significant role in metastasis (Wang et al., 2004). A recent study by Iyer and Pumiglia (2005), on immortalized keratinocytes, proposed a model about the role of \(\alpha_3\beta_1\) integrin in the progression of squamous cell and other carcinomas. According to that model, \(\alpha_3\beta_1\) is required for the stabilization of matrix metalloproteinase-9 (MMP-9) mRNA in response to Ras/MEK/ERK mediated transcriptional activation of MMP-9 gene. In the absence of \(\alpha_3\beta_1\), transcriptional activation by Ras/MEK/ERK signaling does not cause enough accumulation of MMP-9 protein because of the rapid degradation of MMP-9 mRNA. Matrix metalloproteinases are secreted as inactive enzymes into the extracellular spaces and become activated by self proteolysis or proteolysis by other MMPs or proteinases (Iyer et al., 2005). Activated MMPs result in tumor progression and invasion through degradation and remodeling of extracellular matrix (Iyer et al., 2005). Natali et al. (1993), detected progressive increase in the expression of \(\alpha_3\beta_1\) integrin during tumor progression in cutaneous malignant melanoma. Further experiment by Melchiori et al. (1995), suggested that increased expression of \(\alpha_3\beta_1\) is critical for melanoma cell progression to an invasive, malignant
phenotype. Their results indicated that $\alpha_3\beta_1$ is preferentially involved in melanoma cell migration and invasion rather than adhesion. The higher expression of $\alpha_3\beta_1$ heterodimer was also observed in human breast carcinoma metastases compared with the corresponding primary tumors (Morini et al., 2000). In vitro, experiment showed that $\alpha_3\beta_1$ integrin is critical for invasion and migration of human breast carcinoma cell lines (Morini et al., 2000). Increased expression of $\alpha_3\beta_1$ has also been observed in an in vivo brain metastatic model established from human non-small cell lung cancer (NSCLC) cell lines, in athymic mice (Yoshimasu et al., 2004). Interaction of the heterodimer $\alpha_3\beta_1$ and its ligand, laminin, plays important roles in the process of brain metastasis of non-small cell lung cancer (Yoshimasu et al., 2004). Takatsuki et al. (2004), demonstrated that $\alpha_3\beta_1$ integrin plays an important role in the adhesion of cancer cells to the peritoneum. Human gastric carcinoma cells showed reduced adhesion to the excised peritoneum when treated with anti-$\alpha_3$ and anti-$\beta_1$ antibodies (Takatsuki et al., 2004). It was suggested that the initial attachment of cancer cells to peritoneal lining mediated by $\alpha_2\beta_1$ and/or $\alpha_3\beta_1$ integrin is an important step toward the subsequent formation of metastasis (Takatsuki et al., 2004).

$\alpha_6\beta_4$ INTEGRIN

$\alpha_6\beta_4$ integrin is a transmembrane receptor protein which mediates cell-cell and cell-extracellular matrix interactions. In normal epithelial cells, it is required for the formation of the adhesive structures called hemidesmosomes (Owens et al., 2003). Involvement of the $\alpha_6\beta_4$ integrin in epithelial cells, promotes cell cycle progression through activation of RAS-MAPK pathway (Mainiero et al., 1997). Falk-Marzillier et al. (1998), reported the biochemical properties of the complex formed between $\alpha_6\beta_4$ integrin and its ligand laminin-5 (Ln-5). According to these investigators, this complex may extend the $\alpha_6\beta_4$-intermediated filament link to the basement membrane, thus preventing epithelia from detachment. Expression of $\alpha_6$ integrin becomes down regulated in neoplastic human breast epithelium compared with normal breast epithelium.
(Damjanovich et al., 1997). Altered expression of \( \alpha_6\beta_4 \) integrin has been observed in N-nitrosomethylbenzylamine (NMBA) induced rat esophageal tumor (Khare et al., 1998). Reduced expression was found both at protein and mRNA level. This differential expression of \( \alpha_6\beta_4 \) integrin may serve as a biomarker in rat esophageal cancer model (Khare et al., 1998). Role of \( \alpha_6\beta_4 \) as a predictive marker for peritoneal dissemination has also been suggested in human gastric cancer (Ishii et al., 2000). Over expression of \( \alpha_6\beta_4 \) integrin was reported in bladder cancer in which the evaluation of the expression of this molecule is important in determining prognostic outcome (Grossaman et al., 2000). Keratinocytes express \( \alpha_6\beta_4 \) integrin suprabasally. This suprabasal expression promotes tumourigenesis, by preventing transforming growth factor \( \beta \)-mediated growth inhibition (Owens et al., 2003). Zhou et al. (2004), reported increased expression of this transmembrane protein in human laryngeal carcinoma cell lines, and suggested that treatment with anti- \( \alpha_6 \) and/or anti-\( \beta_4 \) specific monoclonal antibodies may reduce the development of laryngeal carcinoma.

**ESOPHAGEAL CANCER**

Cancer of the esophagus is the ninth most common cancer in the world (Kuwano et al., 2005). The prognosis of this cancer is poor and the 5-year survival rate is less than 10% (McCabe et al., 2005). According to von Rahden et al. (2005), survival of the patients with preoperative chemotherapy is higher than without preoperative chemotherapy. The highest risk areas include so-called Asian esophageal cancer belt of Iraq, Iran, western and northern China, Hong Kong, Japan, as well as southwestern Africa, France and parts of South America (Kuwano et al., 2005). Approximately 11,000 - 13,000 new cases of esophageal cancer are diagnosed each year in the United States (see A in appendix). Figure 3 describes the growing incidences of esophageal cancer in the United States of America.
Figure 3: (see A in appendix).
TYPES OF ESOPHAGEAL CANCER

Esophageal cancer is of two types: squamous cell carcinoma (SCC) and adenocarcinoma. The incidence of adenocarcinoma has been currently increasing particularly in the Western population (Kuwano et al., 2005).

SQUAMOUS CELL CARCINOMA

This is the cancer of the thin, flat squamous cells that form the lining of the esophagus. This cancer is frequently found in the upper and middle part of the esophagus. There are two major risk factors for the development of the squamous cell carcinoma of esophagus: alcohol and tobacco smoking which exert synergistic effects (Kuwano et al., 2005). Esophageal dysplasia is the chief lesion of the esophageal SCC (Kuwano et al., 2005). Esophageal squamous cell carcinoma develops gradually from mild to severe dysplasia, to carcinoma in situ and finally to
invasive carcinoma ((Kuwano et al., 2005). According to the National Cancer Institute, the risk factors of the esophageal squamous cell carcinoma include the following:

- Use of tobacco.
- Excessive Alcohol drinking.
- Malnourishment.
- Human papilloma virus (HPV) infection.
- Tylosis and achalasia.
- Drinking of very hot liquids regularly. (see B in appendix).

**ADENOCARCINOMA**

Most of the esophageal cancers are adenocarcinoma which begins in the lower esophagus. The major risk factor for esophageal adenocarcinoma is gastroesophageal reflux disease (GERD). Adenocarcinoma starts developing in the glandular tissue. Metaplastic Barretts’s mucosa causes esophageal adenocarcinoma due to continuous injury from GERD (Lagergren et al., 1999; Spechler, 2002). According to the National Cancer Institute, the risk factors for the esophageal adenocarcinoma include the following:

- Gastroesophageal reflux disease.
- Barrett’s esophagus.
- Being overweight.
- History of drug use to relax lower part of the esophagus. (see B in appendix).
SYMPTOMS OF ESOPHAGEAL CANCER

According to the Mayo Foundation for Medical Education and research (see C in appendix), the general symptoms of the esophageal cancer are:

a) difficulty in swallowing,
b) weight loss,
c) pain either in throat, mid-chest or between shoulder blades, and
d) chronic cough.

STAGES OF ESOPHAGEAL CANCER

The stages of esophageal cancer are as follows:

- **Stage 0**: (Carcinoma in situ): At this stage, the cancer is noninvasive.
- **Stage I**: At this stage, the top layer cells of the esophagus become cancerous.
- **Stage II**: At this stage, cancerous cells invade deeper layer of the esophagus lining and also spread to adjacent lymph nodes.
- **Stage III**: Further invasion of the cancerous cells into the deep of the esophageal wall and also adjacent tissue.
- **Stage IV**: At this stage, the cancer has metastasized, i.e. becomes spread into other parts of the body. (see D in appendix).

GENETIC CHANGES IN ESOPHAGEAL CANCER

*p53*: Research studies on the genetic alterations in the esophageal cancer and its relationship with the progression of cancer, early detection, and identification of various genetic biomarkers are being conducted worldwide. Parenti et al. (1995), and Kitamura et al. (1996), observed the mutation of p53 in the early stage of esophageal squamous cell carcinoma and its relation with the progression of cancer. A significant alteration of p53 mutations were observed
in the patients with esophageal cancer who smoke and consume excessive alcohol (Saeki et al., 2000). Overexpression of p53 protein due to genetic alterations have been found to induce production of anti-p53 serum antibody, which could be used as a useful tool for the detection of the esophageal cancer (Kato et al., 2000; Shimada et al. 1998, 2002).

\textit{p21}: p21 (waf/CIPI) is an important regulator of cell-cycle. This gene is a cyclin dependent kinase inhibitor (Kuwano et al., 2005). Bahl et al. (2000), reported the polymorphisms of this gene in the esophageal squamous cell carcinoma and suggested that this may play an important role in esophageal tumorigenesis.

\textit{p16}: p16 gene codes for a tumor suppressor protein which inhibits the function of the Cyclin D1/CDK4 and CDK6 (Kuwano et al., 2005). Tokugawa et al. (2002), observed silence mutation of this gene. The mutation occurs either through methylation or loss of heterozygosity (LOH) in the early stages of esophageal squamous cell carcinoma. According to Nie et al. (2004), and Hibi et al. (2001), detection of methylation in the CpG islands of the p16 gene may serve as a useful biomarker.

\textit{Cyclin}: Cyclins are cell cycle regulatory proteins that form complex with cyclin-dependent kinase. This complex regulates the cell cycle by phosphorylating its targets (Kuwano et al., 2005). There are different types of cyclins, out of which Cyclin A and B regulate G2-M phase transition of the cell cycle, while Cyclin D1 and E controls the G1-S check point (Draetta et al.,1989; Lew et al., 1991). Wang et al. (1996), reported overexpression of Cyclin D1 on NMBA induced rat esophageal cancer. Overexpression of Cyclin D1 has also been observed in human squamous cell carcinoma (Shamma et al., 2000). Watanabe et al. (1999), studied on the role of Cyclin D1 overexpression in cell transformation. Overexpression of Cyclin E has also been observed in esophageal squamous cell carcinoma (Matsumoto et al., 1999).
**COX-2:** Cyclo-oxygenase (COX) is a rate-limiting enzyme for prostanoid synthesis (Kuwano et al., 2005). Tsuji et al. (1998), reported the role of COX in cancer growth via angiogenesis while Sawaoka (1999), demonstrated that COX inhibitors can suppress tumor growth by preventing the expression of angiogenic factors. Overexpression of COX-2 has been found in esophageal adenocarcinoma (Zimmermann et al., 1999). Nozoe et al. (2005), reported the expression of COX-2 in esophageal squamous cell carcinoma and correlated the degree of expression with the progression of the tumor.

**DETERMINATION OF VARIOUS MARKERS IN ESOPHAGEAL CANCER**

In order to prevent the cancer progression, it is very important to detect the cancer at early stage before it spreads to other organs. Detection of effective genetic and protein markers could help early detection of the disease. Khare et al. (1998), reported the role of α-catenin and E-cadherin as effective indicators, of the invasive potential of the esophageal cancerous cells in rat. Decreased expression of GST-pi gene at the mRNA level was observed in some cases of esophageal cancer. This reduction in expression was reported as an early event of esophageal carcinogenesis (Fu et al., 1999). Expression of cyclin D1 was observed in patients who underwent esophagectomy. Results revealed lower survival of the patients who have higher expression of cyclin D1 (Nagasawa et al., 2001). Current studies on vascular endothelial growth factor-C (VEGF-C) suggest its role in the spread of several cancers to the lymphatic system (Noguch et al., 2002). According to Noguch et al. (2002), VEGF-C might play an important role in metastasis of esophageal squamous cell carcinoma. Reduced expression of transforming growth factor-beta receptors (TGF-beta R-I and TGF-beta RII) were observed in esophageal squamous cell carcinoma. This reduction was correlated with the depth of invasion and lymph node metastasis (Fukai et al., 2003). Sud et al. (2005), reported that altered expression of a G-protein coupled receptors GPCR56, is an early event in the esophageal tumorigenesis. L-type-
amino acid transporter1 (LAT1) also plays an important role in cellular proliferation. Increased expression of LAT1 has been observed in esophageal squamous cell carcinoma (Kobayashi et al., 2005).

**INTEGRINS IN ESOPHAGEAL CANCER**

The essential processes for the progression of cancer are invasion, differentiation and migration. Integrins play important roles in the regulation of cancer cell growth, survival, migration, and invasion. Therefore detailed information about the molecular mechanisms by which integrins act at each step of the cancer metastasis would be extremely helpful to combat the disease. Cytokines stimulate cancer cell growth, which results in increased expression of the β1 integrin family. According to Sato et al. (1996), heparin-binding epidermal growth factor (HB-EGF) increases the expression of α2β1 and α3β1 integrins in esophageal cancer cells (TE-1 and T.Tn), which facilitates transmigration of cancer cells to extravascular tissue. Normal cell-cell interactions have to be down-regulated for a tumor cell arising from epithelial tissue in order to begin invasion (Dedhar, 1990). Miller et al. (2001), found that human esophageal squamous cell carcinoma cell lines, WHOC1, WHCO3, WHCO5 and WHCO6 express lower level of α2, β1 integrins in comparison to the normal esophageal tissue. The down-regulation of these molecules was correlated with the invasion and the metastatic abilities of these cell lines. This study also found strong expression of αV integrin by these cell lines, which was completely absent in the normal esophageal cells. Therefore, αV integrin could serve as an indicator of transformed phenotype in esophageal squamous cell carcinoma. Up-regulation of integrin α3, α6 and β4 genes were observed in human esophageal squamous carcinoma cell lines compared to normal esophageal epithelium (Hu et al., 2001). Takayama et al. (2003), observed reduced expression of β1 integrin along with E-cadherin, CD 44, and CD 44v6 in the lymph node metastasis in
comparison with the primary human esophageal tumor. This reduction was correlated with the progression of lymph node metastasis.

Recently, there has been a growing interest in the investigation of various molecular markers of esophageal carcinoma, as a potential diagnostic, prognostic as well as therapeutic tool. Monoclonal antibodies like Trastuzumab for breast cancer (www.cancer.org, 2005), Bevacizumab for colorectal cancer (www.cancer.org, 2005), Alemtuzumab for Chronic lymphocytic leukemia (CLL) (www.cancer.org, 2005) have already been approved for the treatment of cancers. Several other monoclonal antibodies are under clinical trials. Monoclonal antibody for clinical applications of human esophageal cancer is yet to be developed. A mouse monoclonal antibody produced (in our laboratory) against human esophageal cancerous cell lines was found to react with the $\alpha_3\beta_1$ integrin (Jamasbi et al., 2003). The main objective of this investigation was to determine the expression levels of $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins in various rat cancerous cell lines. Monoclonal antibody, 5A, produced against a tumorigenic rat esophageal cell line, RE-B2T (Jamasbi et al., 1992), and monoclonal antibody against $\alpha_6\beta_4$ integrin (gift of Dr. S.J. Kennel from Oak Ridge National Laboratory, USA) were used in the present investigation. The experiments were performed using tumorigenic, non-tumorigenic and normal rat cell lines. The expression of the integrin was determined both at the mRNA and protein level and analyzed statistically. Since integrins play important roles in cancer progression and metastasis, information about the level of expression of these molecules at the various stages of esophageal cancer would help the scientific community to understand the mechanisms of progression of the esophageal cancer and in designing a new therapeutic approach.
MATERIALS AND METHODS

CELL LINES

Rat esophageal cell lines RE-B2T, RE-79, RE-149, RE-C1T, tracheal cell line BP3, C18, and human esophageal squamous cell carcinoma cell line TE5 were used in this study. *In vitro* treatment of rat esophagus tissue with N-nitrosobenzylmethylamine (NMBA) produced an epithelial cell outgrowth, and a cell line, B-2 was derived from that outgrowth (Stoner et al. 1982, 1989). Inoculation of B-2 cells into newborn syngenic rat produced a tumor, and RE-B2T and RE-C1T cell lines were developed from that tumor. RE-149, RE-79 and RE-282 are nontumorigenic cell lines with the properties of spontaneously transformed cells (Stoner et al., 1989). BP3 is a tracheal squamous cell carcinoma cell line, and C18 is a non-tumorigenic tracheal cell line. All rat cell lines were cultured in PFMR-4 medium with 0.1 mM calcium chloride (Biofluids, Inc., Rockville, MD), 1.0 µM hydrocortisone (Sigma Chemicals, St. Louis, Mo), 1.0 µM phosphoethanolamine (Biofluids, Inc., Rockville, MD), 2.0 mM L-glutamine (Invitrogen Corporation, Grand Island, NY), 5 ng/ml epithelial growth factor (EGF) (Sigma Aldrich, St. Louis, MO)), 5µg/ml insulin, 5µg/ml transferrin (Sigma Chemicals), 25 µg/ml gentamycin sulfate (Fisher Scientific) and 2% fetal bovine serum (FBS) (Hyclone Lab., Logan, UT) supplements. The completed medium was referred as 79S medium (Stoner 1989). The cells were cultured in 100 x 20 mm tissue culture dish (Corning Incorporated, NY,USA) at 37°C in a 5% incubator with 100% humidity. They were passaged at 80% confluency. To passage, the cells were washed once with calcium and magnesium- free phosphate-buffered saline (PBS). Washed cells in tissue culture dish were then treated with 2 ml of trypsin-EDTA solution (0.4% trypsin and 0.02 % EDTA in PBS) for 5-10 minutes in a 37°C incubator. Trypsin treated cells were then washed once with the cell culture medium and transferred into new tissue culture dishes, at approximately 10^5 cells per dish. Cultured cells were frozen periodically in medium containing
10% dimethylsulfoxide (DMSO, Sigma Chemicals) and stored at -80°C.

Normal rat tracheal, and kidney cells were used as controls in this study. Primary cultures of normal cells were established from Fisher 344 rats (Jamasbi and Nettlesheim 1977; Babcock et al. 1983). The trachea and kidney were removed aseptically from normal rats. The organs were then cut into small pieces with surgical scissors and placed into tissue culture dishes containing 79S medium. The dishes were incubated in 5% CO₂ incubator at 37°C with 100% humidity. The cells were continued to grow until the dish surface was covered by the monolayer of cells. Cultured cells were harvested by trypsin-EDTA treatment, washed with PBS, plated in 96-well plates (Corning Incorporated, NY, USA), and fixed for subsequent experiments.

Human cell lines were cultured in Dulbecco’s Modified Eagle’s medium (Invitrogen,) supplemented with 5% fetal bovine serum (HyClone Lab., Logan, UT), 2.0mM L-glutamine (Invitrogen Corporation, Grand Island, NY), and penicillin (100 µU/ml) and streptomycin (100 µg/ml).

**MONOCLONAL ANTIBODY PREPARATION**

The monoclonal antibody, designated mAb-5A (IgG1) used in this study was produced against the tumorigenic rat esophageal epithelial cell line, RE-B2T (Jamasbi, 1992). The antibody was concentrated from supernatants of hybridoma culture by the ammonium sulphate precipitation method (Jamasbi, 1992). Hybridoma cells were cultured in Dulbecco’s modified Eagle’s medium. The medium was supplemented with 2 mM L-glutamine, 100µg/ml streptomycin (Invitrogen Corporation, Grand Island, NY), 100µU/ml penicillin (Invitrogen Corporation, Grand Island, NY) and 20% FBS. Approximately 500 ml of supernatant was collected from the medium containing growing hybridoma cells by centrifugation for 7 minutes at 1500x g (Beckman Instruments Inc., USA). The supernatant was then mixed with equal volume of saturated ammonium sulfate (Fisher Scientific) in phosphate buffered saline. The
mixture was then centrifuged at 4,500x g for 10 minutes at 20 to 23°C (Sorvall RC 5C, CT, USA). After centrifugation, pellet was collected and dissolved in 40 ml of 50% saturated ammonium sulfate solution. The antibody suspension was centrifuged again for 10 minutes at the same temperature and speed. The supernatant was collected and dialyzed against PBS at 4°C for 36 hours with three changes of PBS at 12 hours interval. Finally, the antibody was dispensed in small aliquots and stored at -20°C.

**PREPARATION OF FIXED CELLS**

Cultured cells were trypsinized and washed in ELISA medium. 79S medium was added to the washed cells and transferred into 96-well tissue culture plate. The cells were grown in a 5% CO₂ incubator at 37°C with 100% humidity. When the cells in each well formed a monolayer, they were washed once with PBS. Cells were fixed with 100µL of 0.1% gluteraldehyde (Sigma Chemicals) in PBS for 10 minutes at room temperature. After washing 3 times with PBS, the cells were incubated with 100 µL of 0.1 M glycine (Sigma Chemicals) in PBS for 5 minutes at room temperature. Finally, the cells in the wells were washed 3 times with PBS and 100 µL of Dulbecco’s Modified Eagle Medium, (supplemented with 1% penicillin-streptomycin and 5% fetal bovine serum) was added into each well. The fixed cells in 96-well plates were stored in a -20°C freezer and used for ELISA experiments.

**DETERMINATION OF THE OPTIMAL REACTIVITY OF THE ANTIBODY**

To determine the optimum reactivity of the monoclonal antibodies, ELISA was performed using various dilutions (1: 100, 1:200, 1: 400, 1: 800, 1: 1600 and 1: 3200) of the primary antibody in ELISA medium. Fixed cells in each well were incubated with 100 µL of the diluted antibody at 37°C for two hours. The cells were washed 3 times with PBS and reacted with 100 µL of β-galactosidase-conjugated goat anti-mouse secondary antibody (Southern Biotech, Birmingham, AL) (1: 500 dilution in ELISA medium) for another 2 hours at 37°C. After
3 times washing with PBS, the cells in each well were incubated for 1 hour at 37°C with 100 µL of substrate solution. Substrate solution was prepared by dissolving 1mg/ml of p-nitrophenyl-β-D-galactopyranoside (Sigma Aldrich, St. Louis, MO) in phosphate buffer (pH 8.0). The yellow color was developed due to enzyme-substrate reactions. The intensity of the color was measured by microplate reader (MR-600 microwell plate reader, Dynatech Lab. Inc.) at optical density of 410 nm.

**DETERMINATION OF THE CHEMICAL CHARACTERISTICS OF THE EPITOPE**

To characterize the chemical composition of the epitope reactive with monoclonal antibody 5A, following experiments were performed.

**PERIODIC ACID TREATMENT**

Ravindranath et al. (1989), and Natio et al. (1990), reported that both sodium m-periodate and periodic acid can cleave the carbohydrate moieties from glycolipid molecules. Fixed cells in 96-well plates were treated with 100µL/well of 10mM, 50mM and 100 mM of aqueous periodic acid (Sigma Chemicals, St. Louis, MO) solutions (in water) at 37°C for 30 minutes. The effect of periodic acid on membrane antigen-antibody reaction was determined by ELISA and compared with untreated cells.

**TRYPSIN TREATMENT**

Fixed cells were exposed to 100 µL/well of 1% trypsin (in PBS, pH 7.2) for 60 minutes at 37°C. The effect of trypsin was then determined by ELISA on both treated and untreated cells.

**PROTEIN EXTRACTION**

Total protein was extracted from the cultured cells and the reactivity of the protein antigen was determined by ELISA using mAb-5A. Briefly, cultured cells were scraped from the plate, washed and exposed to 1 ml of lysate buffer {2.5 ml PBS, 0.25 ml 5% NP40 and 20 µL of 10mg/ml Phenylmethylsulfonyl Flouride (Sigma Chemicals) in isopropyl alcohol} for 5
minutes. The mixture was then sonicated (Branson 220 Sonicator, Branson Cleaning Equipment Co.) for 15 seconds. The mixture was then centrifuged (5414 centrifuge, Eppendorf) at maximum speed for 5 minutes and the supernatant was collected. The supernatant was then sonicated for 15-20 seconds. Finally, the mixture was centrifuged for 10 minutes at 10,000x g. The supernatant was collected and the protein concentration in the supernatant was determined by BCA protein assay method (Smith, 1985).

**DETECTION OF PROTEIN ANTIGEN**

To compare the level of protein antigen expression between tumorigenic (RE-B2T) and non-tumorigenic (79) cell lines, 16 µg of extracted protein was attached into the wells of 96-well plates by drying at room temperature under a negative pressure hood. Attached protein was then fixed with 0.1% glutaraldehyde and 0.1 M glycine. The expression was determined by ELISA.

**RT-PCR**

Total RNA was isolated from various cell lines using High Pure RNA tissue Kit (Roche Applied Science, Penzberg, Germany). RNA was isolated from more than 10^6 cells of each cell line according to the manufacturer’s instructions. Briefly, cultured cells were homogenized, and lysed with lysis/binding buffer and centrifuged at 10,000x g for 2 minutes. The supernatant was collected and mixed with 200 µL of absolute ethanol. The mixture was then transferred into high pure filter tube (Roche Applied Science, Penzberg, Germany) and treated with 100 µL of DNase incubation buffer (supplied in the Kit) for 15 minutes at 15 to 25°C. After washing with wash buffers, the column from the filter tube was transferred into a nuclease-free 1.5 mL centrifuge tube (Eppendorff). To elute the extracted RNA, 100 µL elution buffer was added to the column and centrifuged at 7500x g for 1 minute. The eluted RNA was collected into the microcentrifuge tube. The concentration of the RNA was determined by a spectrophotometer (Beckman DU 640, USA) at 260 nm absorbance. Reverse transcription and PCR were performed with gene-specific
primers using Qiagen One Step RT-PCR Kit (Qiagen Inc., Valencia, CA). For RT-PCR, 5 ng of DNA-free total RNA with oligo primers were used. The reaction was performed in 50 µL volume with 1µL of 100 mM of each primer pair. A master mixture was prepared using 10 µL of 5x buffer solution, 2µL of dNTP mix, and 2µL of RT-PCR enzyme mix which contains Omnistcript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStar Taq DNA polymerase. The entire RT-PCR reaction was performed according to the manufacturer’s instruction. PCR was carried out in a thermalcycler (Eppendorf Mastercycler Gradient). The primer pairs used for β1 integrin and β-actin are as follows: β1 integrin:

F5`GACCTGCCTTGGTGTTGCTGTGC3', and R5`GGCAACCACGCCTGCTACAAT3`
(Yashpal, 2005), β actin: F5`GACGGGGTCACCCACACTGTGCCCATCTA3` and
R5`CTAGAAGCATTTGCGGTGCACGATGGAGG3`. All primers were purchased from Opern Biotechnologies, Inc. (Huntsville, AL). PCR conditions were 50°C for 30 minutes for reverse transcription, 15 minutes at 95°C for initial PCR activation, 94°C for 60 seconds, 90 seconds at 55°C, and 72°C for 2 minutes. Samples were subjected to 30 cycles both for β1-integrin and β-actin with final extension for 10 minutes at 72°C. β-actin used as positive control gene. Other control includes RT-PCR reaction mixture with no total RNA. The amplified products were analyzed on a 1.8% agarose gel, and visualized by ethidium bromide staining.
RESULTS

OPTIMAL REACTIVITY OF THE MONOCLONAL ANTIBODY 5A

ELISA result showed that optimal reactivity of the monoclonal antibody 5A is at a 1:200 dilution. This dilution of the antibody was used in the subsequent experiments.

REACTIVITY OF mAb-5A WITH DIFFERENT RAT CELL LINES

ELISA results showed that mAb-5A reacted differentially with the cell lines used in this study (Table 1 and Fig. 5). There was no or very little reactivity with rat fibroblast cell lines. Concentrated soup of the cultured myeloma cell line, SP2/0, was used as control in this experiment. The soup of SP2/0 did not react with any of the above cell lines in ELISA.

Expression of $\alpha_3\beta_1$ integrin was comparatively higher in non-tumorigenic esophageal cell lines, RE-149, and RE-79, than tumorigenic esophageal cell lines RE-B2T and RE-C1T. Normal kidney and tracheal cell lines also showed higher expression of $\alpha_3\beta_1$ integrin than RE-B2T. There was a little difference in the expression of $\alpha_3\beta_1$ integrin between RE-C1T and RE-79. It is plausible that RE-C1T cells represent early stage of tumorigenicity. Higher expression of $\alpha_3\beta_1$ integrin on non-tumorigenic cell lines than on normal cells suggests that increased integrin expression starts during the alteration of cell line. Increased expression of integrin facilitates the adhesion of the cells while reduction aids in the metastasis. The differences in the expression of $\alpha_3\beta_1$ integrin on various cell lines were found to be significant by statistical analysis (P-0.00068, ANOVA).
TABLE 1: Reactivity of mAb-5A with various rat cell lines \(^{(a)}\)

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>ABSORBANCE AT 410 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE-B2T</td>
<td>0.340 ± 0.038</td>
</tr>
<tr>
<td>RE-79</td>
<td>0.648 ± 0.031</td>
</tr>
<tr>
<td>RE-149</td>
<td>0.783 ± 0.021</td>
</tr>
<tr>
<td>RE-C1T</td>
<td>0.628 ± 0.018</td>
</tr>
<tr>
<td>TRACHEA</td>
<td>0.418 ± 0.008</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>0.428 ± 0.069</td>
</tr>
<tr>
<td>FIBROBLAST</td>
<td>0.063 ± 0.014</td>
</tr>
</tbody>
</table>

(a) Cells were grown in 96-well plates and fixed with 0.1% glutaraldehyde. ELISA was performed with mAb 5A at 1:200 dilution. Experiments were performed in triplicates. The optical density was measured at 410 nm using a microwell plate reader.

Figure 5: Determination of the expression of $\alpha_3\beta_1$ integrin on a panel of rat cell lines. Bars represent standard errors.
**REACTIVITY OF ANTI- \( \alpha_6\beta_4 \) MONOCLONAL ANTIBODY WITH VARIOUS RAT CELL LINES**

In addition to \( \alpha_3\beta_1 \) integrin, ELISA results showed that both tumorigenic (RE-B2T) and non-tumorigenic rat esophageal cell lines (RE-79 and RE-149) express \( \alpha_6\beta_4 \) integrin (Table 2 and Fig.6). Expression of \( \alpha_6\beta_4 \) integrin molecule was higher in RE-79 than RE-B2T. Normal trachea and kidney cell lines also reacted with anti- \( \alpha_6\beta_4 \) integrin antibody. The expression of \( \alpha_6\beta_4 \) integrin on fibroblast cells was very low compared to other cell lines. The ELISA results also detected higher expression of \( \alpha_6\beta_4 \) integrin on the non-tumorigenic tracheal cell line, C18 than tumorigenic cell line BP3. Rat monoclonal anti-\( \alpha_6\beta_4 \) integrin antibody was also found to react slightly with human esophageal carcinoma cell line, TE-5. The differences in the expression of \( \alpha_6\beta_4 \) integrin on various cell lines were found to be significant by statistical analysis (\( P=0.00057 \), ANOVA).

**TABLE 2: Reactivity of anti-\( \alpha_6\beta_4 \) antibody with various cell lines \(^{(a)} \)**

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>ABSORBANCE AT 410 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE-79</td>
<td>0.345 ± 0.015</td>
</tr>
<tr>
<td>RE-B2T</td>
<td>0.309 ± 0.030</td>
</tr>
<tr>
<td>RE-C18</td>
<td>0.416 ± 0.053</td>
</tr>
<tr>
<td>BP3</td>
<td>0.228 ± 0.044</td>
</tr>
<tr>
<td>TE5</td>
<td>0.234 ± 0.009</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>0.232 ± 0.036</td>
</tr>
<tr>
<td>TRACHEA</td>
<td>0.439 ± 0.049</td>
</tr>
<tr>
<td>FIBROBLAST</td>
<td>0.114 ± 0.003</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Cells were grown in 96-well plates and fixed with 0.1% glutaraldehyde. ELISA was performed with anti- \( \alpha_6\beta_4 \) antibody at 1:200 dilution. Experiments were performed in triplicates. The optical density was measured at 410 nm using a microwell plate reader.
Figure 6: Determination of the expression of integrin $\alpha_6\beta_4$ on a panel of rat cell lines. Bars represent standard errors.

PERIODIC ACID TREATMENT

Periodic acid treatment of RE-C1T, RE-149, and normal trachea and kidney cell lines with various concentrations of periodic acid abolished their reactivity with the monoclonal antibody 5A (Fig. 7-10 and table 3-6). The results indicate carbohydrate composition of the epitope. The ELISA results were analyzed statistically by paired t-test.
TABLE 3: Reactivity of periodic acid treated and untreated RE-CIT cells with mAb-5A (a)

<table>
<thead>
<tr>
<th>Concentration of the acid (mM)</th>
<th>Untreated</th>
<th>Treated</th>
<th>% of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.09 ± 0.087</td>
<td>0.038 ± 0.017</td>
<td>96.51</td>
</tr>
<tr>
<td>50</td>
<td>1.344 ± 0.434</td>
<td>0.034 ± 0.017</td>
<td>97.47</td>
</tr>
<tr>
<td>100</td>
<td>1.118 ± 0.132</td>
<td>0.047 ± 0.018</td>
<td>95.79</td>
</tr>
</tbody>
</table>

(a) CIT cells were grown in a 96-well plate and fixed with 0.1% glutaraldehyde. Fixed cells were incubated with 10, 50 and 100 mM of periodic acid for 30 minutes at 37°C. ELISA was performed on both periodic acid treated and untreated cells. The experiments were performed in triplicates using mAb-5A at 1:200 dilution, the optical density was measured at 410 nm in a micro well plate reader.

(b) The percentage of reduction in the antigen-antibody reaction was determined by the following formula:

\[
\% \text{ of reduction} = \frac{\text{O.D (untreated)} - \text{O.D. (treated)}}{\text{O.D (untreated)}} \times 100.
\]

Figure 7: Effect of periodic acid treatment on RE-C1T cell line. Bars represent standard errors.
TABLE 4: Reactivity of periodic acid treated and untreated RE-149 cells with mAb-5A (a)

<table>
<thead>
<tr>
<th>Concentration of the acid (mM)</th>
<th>Untreated</th>
<th>Treated</th>
<th>% of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.197 ± 0.295</td>
<td>0.035 ± 0.004</td>
<td>97.07</td>
</tr>
<tr>
<td>50</td>
<td>1.064 ± 0.347</td>
<td>0.039 ± 0.005</td>
<td>96.33</td>
</tr>
<tr>
<td>100</td>
<td>0.665 ± 0.002</td>
<td>0.028 ± 0.007</td>
<td>95.78</td>
</tr>
</tbody>
</table>

(a) RE-149 cells were grown in a 96-well plate and fixed with 0.1% glutaraldehyde. Fixed cells were incubated with 10, 50 and 100 mM of periodic acid for 30 minutes at 37°C. ELISA was performed on both periodic acid treated and untreated cells. The experiments were performed in triplicates using mAb-5A at 1:200 dilution, the results are expressed as optical density measured at 410 nm using a micro well plate reader.

(b) The percentage of reduction in the antigen-antibody reaction was determined by the following formula:

\[ \text{% of reduction} = \frac{\text{O.D (untreated)} - \text{O.D (treated)}}{\text{O.D (untreated)}} \times 100. \]

Figure 8: Effect of periodic acid treatment on RE-149 cell line. Bars represent standard errors.
TABLE 5: Reactivity of periodic acid treated and untreated normal rat kidney cells with mAb-5A (a)

<table>
<thead>
<tr>
<th>Concentration of the acid (mM)</th>
<th>Untreated</th>
<th>Treated</th>
<th>% of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.435 ± 0.023</td>
<td>0.076 ± 0.008</td>
<td>82.52</td>
</tr>
<tr>
<td>50</td>
<td>0.375 ± 0.007</td>
<td>0.090 ± 0.006</td>
<td>76.0</td>
</tr>
<tr>
<td>100</td>
<td>0.352 ± 0.042</td>
<td>0.096 ± 0.001</td>
<td>72.72</td>
</tr>
</tbody>
</table>

(a) Normal rat kidney cells were grown in a 96-well plate and fixed with 0.1% glutaraldehyde. Fixed cells were incubated with 10, 50 and 100 mM of periodic acid for 30 minutes at 37°C. ELISA was performed with both periodic acid treated and untreated cells. The experiments were performed in triplicates using mAb-5A at 1:200 dilutions, the results are expressed as optical density measured at 410 nm using a micro well plate reader.

(b) The percentage of reduction in the antigen-antibody reaction was determined by the following formula:

\[
\% \text{ of reduction} = \frac{O.D \text{ (untreated)} - O.D \text{ (treated)}}{O.D \text{ (untreated)}} \times 100.
\]

Figure 9: Effect of periodic acid treatment on normal rat kidney cells. Bars represent standard errors.
TABLE 6: Reactivity of periodic acid treated and untreated normal rat tracheal cells with mAb-5A (a)

<table>
<thead>
<tr>
<th>Concentration of the acid (mM)</th>
<th>Untreated</th>
<th>Treated</th>
<th>% of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.112 ± 0.029</td>
<td>0.083 ± 0.008</td>
<td>92.53</td>
</tr>
<tr>
<td>50</td>
<td>1.19 ± 0.141</td>
<td>0.061 ± 0.017</td>
<td>94.87</td>
</tr>
</tbody>
</table>

(a) Normal rat tracheal cells were grown in a 96-well plate and fixed with 0.1% glutaraldehyde. Fixed cells were incubated with 10, 50 and 100 mM of periodic acid for 30 minutes at 37°C. ELISA was performed with both periodic acid treated and untreated cells. The experiments were performed in triplicates with mAb-5A at 1:200 dilutions, the results are expressed as optical density measured at 410 nm using a micro well plate reader.

(b) The percentage of reduction in the antigen-antibody reaction was determined by the following formula:

\[
\% \text{ of reduction} = \frac{\text{O.D (untreated)} - \text{O.D. (treated)}}{\text{O.D (untreated)}} \times 100.
\]

Figure 10: Effect of periodic acid treatment on normal rat tracheal cells. Bars represent standard errors.
EFFECTS OF TRYPsin TREATMENT ON ANTIGEN-ANTIBODY BINDING

The antigenic epitope was found to be sensitive to proteinase treatment. Considerable reductions in antibody reactivity were observed after trypsin treatment (table 7).

TABLE 7: Effects of trypsin treatment on various cell lines (a)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trypsin treated</th>
<th>Untreated</th>
<th>% of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE-149</td>
<td>0.338 ± 0.028</td>
<td>0.642 ± 0.022</td>
<td>47.35</td>
</tr>
<tr>
<td>RE-79</td>
<td>0.168 ± 0.037</td>
<td>0.682 ± 0.055</td>
<td>75.36</td>
</tr>
<tr>
<td>C1T</td>
<td>0.131 ± 0.007</td>
<td>0.248 ± 0.020</td>
<td>47.17</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>0.079 ± 0.021</td>
<td>0.76 ± 0.008</td>
<td>89.60</td>
</tr>
<tr>
<td>TRACHEA</td>
<td>0.498 ± 0.012</td>
<td>0.638 ± 0.066</td>
<td>21.94</td>
</tr>
</tbody>
</table>

(a) Cells were grown in 96-well plates and fixed with 0.1% glutaraldehyde. Fixed cells were treated with 100 µL of 1% trypsin for one hour at 37°C. Effect of trypsin on antigen was determined by ELISA. The experiments were performed in triplicates using mAb-5A at 1:200 dilutions.

(b) The percentage of reduction in the antigen-antibody reaction was determined by the following formula:

\[
\% \text{ of reduction} = \frac{\text{O.D (untreated)} - \text{O.D (treated)}}{\text{O.D (untreated)}} \times 100.
\]

DETECTION OF $\beta_1$ INTEGRIN EXPRESSION AT mRNA LEVEL

Expression of the $\beta_1$ integrin was also evaluated at the mRNA level. The RT-PCR analysis suggests the increased expression of $\beta_1$ integrin in the non-tumorigenic cell lines than the tumorigenic cell line (B-2T). This result supports the result obtained by the ELISA.
experiment. This experiment also detected the expression of β1 integrin subunit in the normal fibroblast cell lines. However, expression of the house keeping gene β-actin was almost equal in all cell lines.

Figure 11: Expression of the transcript encoding β1 integrin and β actin in various rat cell lines. (a) Total RNA was isolated from the cultured cells using an RNA extraction kit. Reverse transcription was performed with β1 integrin gene specific primers. β-actin gene was used as control. cDNA was amplified for 30 cycles, both for the samples and control. PCR products were analyzed on a 1.8% agarose gel and visualized with ethidium bromide.
**DETECTION OF PROTEIN ANTIGEN**

**TABLE 8:** Reactivity of the protein antigen with mAb-5A \(^{(a)}\).

<table>
<thead>
<tr>
<th></th>
<th>RE-B2T</th>
<th>RE-79</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.115 ± 0.017</td>
<td>0.221 ± 0.002</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Total protein was extracted from the cultured cells using lysate buffer. Concentration of the protein was determined by BCA method. Equal amounts of proteins were attached into the wells of 96-well plated, air dried and fixed with 0.1% glutaraldehyde. ELISA was performed in triplicates and the results were expressed as optical density at 410 nm using a micro well plate reader. Monoclonal antibody 5A was used at 1:200 dilutions.

Figure 12: Expression of protein antigen on RE-B2T and RE-79 cell lines. Bars represent standard errors.

The results showed that there was considerable reduction of the expression of the antigen at the protein level in the tumorigenic cell line, RE-B2T than in non-tumorigenic cell line, RE-79.
DISCUSSION

Increased migration and invasion into extracellular matrices is a key factor in the progression of cancer to a malignant and metastatic phenotype. Careful regulation of both cell-cell and cell-extracellular matrix interactions are essential for the normal functions and tissue organizations by epithelial cells. In malignant cancerous cells, adhesion molecules, such as integrins, which are responsible to mediate these interactions, are deregulated. Following malignant transformation, the expression and functions of integrin molecules are altered, which causes increased invasiveness and metastasis of the transformed cells (Clezardin, 1998). In order to metastasize, tumor cells need to detach from neighboring cells, degrade the basement membrane and penetrate into the interstitial stroma (see review: Guo and Giancotti, 2004).

Integrin molecules bind the extracellular matrix to the cytoskeleton at focal adhesion junctions, and facilitate cell migration (Huttenlocher et al., 1995). Changes in the integrin expression as well as the loss of adhesion are therefore essential for the cancer cells to proliferate, migrate and invade. In this study, lower expression of $\alpha_3\beta_1$ integrin was found on tumorigenic esophageal cell line (RE-B2T) than the non-tumorigenic cell lines (RE-149, RE-79; Table 1 and Fig. 5). Among the cell lines used in this study, decreased expression of both $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrin was observed in the more invasive cell line, RE-B2T, than other cell lines (Table 1, 2 and Fig 5, 6). This reduction may indicate that tumor progression could be facilitated by down-regulation of integrins in esophageal carcinoma. Differential expression of the integrin molecules were also observed in the non-tumorigenic cell lines (Table 1, 2 and Fig 5, 6). Similar findings have been reported by other investigators. In colon carcinoma, reduced expression of $\alpha_3\beta_1$ integrin has been observed, and it was suggested that this reduction could contribute to the migratory ability of the tumor cells (Stallmach et al., 1992). Other studies suggested that down-regulation of $\alpha_3\beta_1$ integrin appears to be associated with malignancy (Giannelli et al., 2002). Guo and Giancotti, (2004),
found that transformed cells have a tendency to lose the expression of the integrins which mainly act as adhesive receptors. Therefore, decreased expression of integrin might facilitate the detachment of the cancerous cells from neighboring cells. Lower expression of other integrin molecule has also been observed in esophageal squamous cell carcinoma. Miller and Veale, (2001), found reduction in the expression of $\alpha_2\beta_1$ integrin in various cell lines established from human esophageal squamous cell carcinoma in comparison to normal esophageal tissue. In the present investigation, reduction in the expression of $\beta_1$ integrin on tumorigenic esophageal cell line, RE-B2T, was also observed by RT-PCR analysis (Fig. 11). Reduction in the expression of $\beta_1$ integrin has been correlated with anchorage independent growth (Suzuki and Takahashi, 1999). This reduction in $\alpha_3\beta_1$ integrin expression therefore might facilitate the RE-B2T cells to overcome the adhesion dependent constraints, and make RE-B2T cells to progress to the tumorigenic stage.

In rat, integrins are expressed at the protein and mRNA level throughout the development (Yashpal et al., 2005). Variation in the expression of $\alpha_3\beta_1$ integrin was also observed at the protein level (table 8 and Fig. 12). Lesser reactivity of the RE-B2T protein with the mAb-5A than RE-79 was observed in this study. This reduced reactivity of the RE-B2T protein therefore supports the result obtained by RT-PCR.

Periodic acid and trypsin treatments reduced or abolished the antigen-antibody reactions in all cell lines (table 3-7 and Fig. 7-10). This change indicates that the reactive epitope contains both carbohydrate and protein moieties

This study also revealed that more invasive rat esophageal carcinoma cell line RE-B2T, expresses lower amount of $\alpha_6\beta_4$ integrin than any other cell lines used in this investigation. According to Giannelli et al., 2002, down regulation of the expression of this integrin molecule facilitates the growth of the tumor. In addition, the role of $\alpha_6\beta_4$ integrin in the progression of
various cancers has been established. Gambaletta et al., (2000), have pointed out that $\alpha_6\beta_4$ integrin plays a significant role in cancer progression by influencing other receptors and key signaling pathway. Survival of the breast carcinoma cells in a stressed condition is also assisted by this molecule (Chung et al., 2002). In skin basal cell carcinoma, both $\alpha_6$ and $\beta_4$ subunits of integrin are down-regulated. This reduction in the expression was correlated with the invasive properties of this tumor (Rossen et al., 1994). Reduced expression of $\alpha_6\beta_4$ integrin has also been observed in breast cancer (Natali et al., 1992). Previous study by Khare et al., (1998), detected reduced expression of $\alpha_6\beta_4$ integrin on rat esophageal papilloma compared with normal and preneoplastic rat esophageal tissue. Decreased expression of this integrin molecule may weaken cell-extracellular matrix attachment, which in turn contributes to the progression of the cancer. According to Rabinovitz and Mercurio (1996), expression of $\alpha_6\beta_4$ integrin might be suppressed for the progression of the tumor cells to the invasive carcinoma. In the present study, expression of $\alpha_6\beta_4$ integrin was found to be lower in the invasive cell line RE-B2T than other cell lines (table 2, Fig. 6). It is possible, that in esophageal carcinoma, initial higher expression of $\alpha_6\beta_4$ integrin promotes cell growth and development, and, the reduced expression of the $\alpha_6\beta_4$ integrin enhances the metastatic capacity.

As growth and progression of tumors are associated with the differential expression of integrins, these molecules could be potential targets for cancer diagnosis, prognosis and therapy. Differential expression patterns of integrins with the progression of the esophageal cancer suggest that these molecules may serve as prognostic markers in esophageal cancers. The results of these study, suggest that expression of both $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins are down-regulated as the cells become more tumorigenic. Further study using in vivo system is needed to understand the roles of these two molecules in development and progression of the esophageal cancer.
REFERENCES


APPENDIX

A.  http://prg.nci.nih.gov/snapshots/Esophageal-Snapshot.pdf: the two graphs were retrieved on June, 17, 2005 from the World Wide Web:


B.  http://www.nci.nih.gov/cancertopics/pdq/screening/esophageal/Patient/page2#Keypoint1, the information was obtained on June 17, 2005.

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