Glycosylation & Disease, 1994, 1, 21–30

Effect of long-term culture of a human laryngeal carcinoma cell line on epitectin production and tumorigenicity in athymic mice

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Epitectin is a high molecular weight mucin-type glycoprotein over-expressed on the surface of human carcinoma cells. In cancer cells, it is proposed to play a protective function and to modulate cell surface properties such as antigenicity and cell adhesion. We have examined the effect of long-term culture on the cell surface expression of epitectin by a human laryngeal carcinoma cell line and the correlation between epitectin expression and tumor production in athymic mice. Indirect immunofluorescence labelling using an epitectin specific monoclonal antibody showed that the level of epitectin on the cell surface was significantly reduced after 78 or more generations in culture. Gel electrophoresis of cell extracts, followed by wheat germ agglutinin and peanut agglutinin overlay analyses, demonstrated similar losses in total cellular epitectin as a result of prolonged passage in culture. The levels of other glycoproteins reacting with wheat germ agglutinin were not significantly altered in high passage cells. Similar results were obtained when HMFG-2 monoclonal antibody was used to probe the levels of cell surface epitectin. In contrast to the above probes, the binding of HMFG-2 to epitectin is independent of glycosylation, therefore it can be concluded that the observed changes are not due to aberration in epitectin glycosylation with increasing passage number but rather due to lack of synthesis of epitectin. The ability of the low epitectin producing H.Ep.2 cells to grow as tumors in athymic mice was reduced compared to the high epitectin producing cells.

Keywords: cell culture, epitectin, mucin-type glycoprotein, nude mice, tumorigenicity

Introduction

Cancer is a disease characterized by dysfunctional cell–cell adhesion and communication, as exhibited by the uncontrolled growth, invasion and metastasis of malignant cells. As components of the cell surface membrane, glycoconjugates are logical mediators of both normal and neoplastic cell–cell interactions. There are many well documented quantitative and qualitative changes in the cell membrane glycoconjugates of human tumors [see reviews 1, 2]. Immunization of mice with human tumor cell membranes or solubilized membrane components has led to the production of numerous monoclonal antibodies which differentiate normal cells from malignant cells. Glycoproteins and glycolipids are the antigens for most of these antibodies [3–5]. We have been investigating the glycoprotein epitectin, defined by the Ca1, Ca2 and Ca3 antibodies, which consists of two sialylglycoproteins of apparent molecular weight 390000 and 350000 [6]. These monoclonal antibodies have

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*This work comprises part of a doctoral thesis by N.A.D. submitted to the Pennsylvania State University.

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been shown to react with a wide range of human cancers [7–9]. Epitectin has been shown to be a mucin-type glycoprotein and its saccharide structures have been characterized [10]. Numerous other monoclonal antibodies, generated using human milk fat globule membranes and breast or other cancer cell components, have been shown to be related to the Ca antibodies [11–13]. It appears that all these antibodies are directed against a family of mucin glycoproteins referred to as MUC1, having similar polymorphic core proteins but different glycosylation [5]. The pattern of glycosylation appears to be tissue-dependent and affected by malignant transformation.

Little is known about the role of mucin-type glycoproteins on the transformed cell surface. Mucins secreted by normal epithelia serve to protect cells from a physicochemical environment that would be destructive; cancer associated mucin-type glycoproteins, such as epitectin, have been proposed to play a similar role [14, 15]. Additional roles for the cancer associated mucin-type molecules may include: increasing tumorigenicity by enabling the cell to escape immune surveillance; and influencing invasion and metastasis by altering cell adhesion and motility. The positive correlation between production of mucin-type glycoproteins and the malignancy of tumor cells provides further evidence for such a role [16, 17]. Furthermore, the rat mammary carcinoma cell line MAT-B1 has been demonstrated to express less ascites sialoglycoprotein, ASGP-1, after long-term culture, and subsequently to be more sensitive to natural killer cell lysis [18, 19]. Other studies show that overexpression of episialin by the normal mammary epithelial cell line (HBL-100) prevents cell aggregation; thus demonstrating that mucin-type glycoproteins can mask cell surface molecules and disturb the interaction of cell surface proteins with macromolecules on adjacent cell membranes [20]. In the present study, we have examined the effect of long-term culture on the cell surface expression of epitectin by H.Ep.2 cells, and the correlation between high and low epitectin expression and tumor production in athymic mice. A preliminary report has been presented elsewhere [21].

Materials and methods

Cell culture

H.Ep.2 cells [22] were obtained from the American Type Culture Collection (CC 23) (Rockville, MD), at approximate passage number 360, and this was considered passage zero for our experiments. CGL1 and CGL3 cells [23] were a generous gift from Professor Eric Stanbridge (University of California, Irvine, CA). Cells were maintained as monolayers in Eagle's minimal essential media (MEM) supplemented with glutamine, sodium pyruvate, non-essential amino acids and 10% (v/v) heat inactivated (56°C, 30 min) fetal bovine serum (Gibco). Growth was at 37°C in 5% CO_2 in 75 cm^2 Corning flasks. These cultures were routinely tested for bacteria, fungi and mycoplasma [24]. The cells were harvested by treatment with versene followed by centrifugation at 1000 ***g*** for 5 min and washed with phosphate buffered saline, 0.14 M Na^+, 0.1 M K^+, 0.006 M PO_4^{3-}, pH 7.2 (PBS). For extraction of epitectin, 10^7 cells were suspended in 750 μl of 50 mM Tris–HCl buffer, pH 8.0 containing 0.2% (w/v) deoxycholate and homogenized by passage through an 18 gauge needle. The homogenate was boiled for 5 min to inactivate enzymes and centrifuged [6]. This supernatant was used for purification of epitectin, immunoprecipitation or sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Metabolically labelling of the cells was done for 48 h with 25 μCi/ml L-[2,3-^{3}H]proline (New England Nuclear, Boston, MA) in amino acid deficient MEM. Cell monolayers were washed with PBS, harvested and then extracted as above.

Immunoprecipitation of epitectin from cell extracts

The Ca2 or HMFG-2 antibodies were used to precipitate epitectin from H.Ep.2 cell extracts as follows: an aliquot of cell extract containing approximately 500 μg of total cell protein was incubated with Protein A–agarose (Pierce, Rockford, IL) and rabbit anti-mouse Ig (Pierce) for preabsorbtion. The supernatant was incubated with 1 μg of antibody overnight at 4°C with end over end shaking. Ca2 antibody was generously provided by Professor H. Harris and Dr M. E. Bramwell (University of Oxford). HMFG-2 antibody [25] was purchased from AMAC, Inc. (Westbrook, ME). Protein A–agarose, precoupled to a rabbit anti-mouse immunoglobulin, was used to precipitate the antigen–antibody complexes. The pellet was washed three times with wash buffer [20 mM Tris–HCl, pH 8.0 containing 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40 and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and then extracted with 50 mM Tris–HCl buffer, pH 8.0 containing 2% (w/v) SDS and 0.1 M dithiothreitol, by boiling at 100°C for 3 min.

Indirect immunofluorescence labelling
About one million cells were incubated in 96 μl of antibody diluent [AD: PBS containing 200 μg/ml goat immunoglobulin and 1% (w/v) ELISA grade bovine serum albumin (BSA)] with 4 μg of either normal mouse immunoglobulin (controls) or one of the monoclonal antibodies: Ca2 or HMFG-2. The cell suspensions were incubated on ice for 30 min, washed three times with 750 μl of wash buffer containing 20% (v/v) fetal calf serum and 2% (w/v) BSA in Ca^{2+}- and Mg^{2+}-free PBS. Cell pellets were resuspended in 99 μl AD containing 1 μg fluorescein-conjugated $F(ab')_2$ fragments of a goat antibody to mouse immunoglobulin (Cappel, Westchester, PA) and incubated on ice for 30 min. Cells were washed as before and suspended in Ca^{2+}- and Mg^{2+}-free PBS for flow cytometric analysis. In controls the test antibody was replaced with normal mouse immunoglobulin.

Flow cytometric analysis
Analysis was performed on an EPICS V flow cytometer/cell sorter (Coulter Electronics) using an argon ion laser (488 nm, 400 nW). Cells were gated on forward angle light scatter (FALS) and 90° light scatter (90° LS) to eliminate cellular debris. Instruments were optimized according to the procedure previously reported [26]. The low and high passage cells were stained with either a specific antibody (test) or normal mouse immunoglobulin (control). Controls were included in each experiment since there was some variation in background binding from one experiment to the next.

Measurement of epitectin in culture media
The level of epitectin in spent culture media was determined by an enzyme-linked immunoabsorbant sandwich assay (ELISA) involving antigen capture by peanut agglutinin (PNA) and measurement by Ca2 antibody binding. The microtiter plates (Dynatech, Alexandria, VA) were coated with PNA, excess sites were blocked with immunoglobulin-free BSA and serially diluted spent culture media was added. The plates were incubated for 2 h at room temperature, washed and then incubated with 50 μl of Ca2 antibody (1 μg/ml in 1.0% BSA–PBS) for 2 h. The wells were emptied, washed and incubated for 2 h with 50 μl of rabbit anti-mouse immunoglobulin antibody conjugated to horse radish peroxidase (1:3000 dilution). The wells were washed five times, incubated with 4.0% (w/v) *o*-phenylenediamine–0.3% (w/v) hydrogen peroxide, and the color intensity measured at 450 nm on a microplate reader.

Electrophoresis of glycoproteins
Protein content of cell extracts was estimated by the Micro BCA protein assay (Pierce). Aliquots of cell extract containing 8 μg protein were boiled in buffer containing 2% SDS and 0.1 M dithiothreitol to reduce disulfide bonds. SDS-PAGE was done on 3.5–12.5% gradient slab gels at 30 mA for 5 h [27]. The gel was stained with 0.05% Coomassie blue in methanol:acetic acid:water (5:1:5) and destained in methanol:acetic acid:water (5:1:5). Glycoproteins were then detected by lectin overlay [28]. Briefly, the destained gel was equilibrated in PBS containing 0.4 M NaCl for 4 h, with two changes. The [^{125}I]lectin solution at 6×10^7 c.p.m./100 ml in PBS 1% BSA (w/v) was added and the gel was incubated overnight at room temperature with gentle shaking. The gel was washed with PBS containing 0.4 M NaCl until washes contained less than 300 c.p.m./100 μl. The gel was shrunk in 50% methanol, dehydrated for 2 h under vacuum at 60°C and subjected to autoradiography at −70°C. [^{125}I]wheat germ agglutinin (WGA) was purchased from ICN Radiochemicals. PNA (E-Y Laboratories) was iodinated using Chloramine-T as described by Burridge [29]. The resulting autoradiograph was scanned on a Molecular Dynamics Laser Densitometer. For detection of metabolically labelled glycoproteins, the destained gels were washed with water and treated with Amplify (Amersham, UK), dehydrated and exposed to Kodak X-OMAT films.

Glycosidase digestion of H.Ep.2 cell surface
Prior to antibody binding, cells were incubated with either 100 mU neuraminidase (*Arthrobacter urefaciens*) (Calbiochem, LaJolla, CA) or a combination of 100 mU neuraminidase and 60 mU of endo-α-*N*-acetylgalactosaminidase for 1 h on ice [30]. Antibody binding and flow cytometry were carried out as above.

Tumor growth
Athymic mice (NCI Frederick Cancer Research Facility) were injected subcutaneously with cells, at doses of 10^4, 10^5 and 10^6 cells per site, four sites (near the limbs) per mouse. Four to six mice were used for each passage number tested. Mice were housed in isolators with sterile cages and bedding. Tumors were measured using vernier calipers at weekly intervals and the mean cross-sectional area was calculated as [31]

$$\frac{\text{Length}}{2} \times \frac{\text{Width}}{2} \times \pi$$

Results

Epitectin expression on the surface of H.Ep.2 cells

In the course of our studies on epitectin, it was noted that the quantities of the radioactively labelled glycoprotein isolated from different batches of cells were quite variable. To investigate the cause of this variability, we quantitated the cell surface epitectin of H.Ep.2 cells from various passage numbers. The level of epitectin at the cell surface was measured by indirect immunofluorescence labelling followed by flow cytometry. The level of Ca2 reactive material on the cell surface significantly decreased with increased passage in culture (Figure 1A–C). After 78 passages, the level of integrated fluorescence of Ca2 binding was decreased by more than two logs of intensity. When the flow cytometry experiments were repeated with high passage cells, which were re-established in culture after storage at −140°C (liquid nitrogen), the level of fluorescence was the same as before freezing down.

The changes in the total cellular levels of epitectin and other glycoproteins produced by the cells of varying passage were then examined. Cells of passage number 12, 96 and 198 were extracted as described in Materials and methods, and equivalent amounts of protein (25 μg) subjected to gel electrophoresis followed by lectin overlay. Epitectin was detected as two bands of apparent molecular weight 350 and 390 kDa, reacting with both WGA and PNA (arrows in Figure 2). The amount

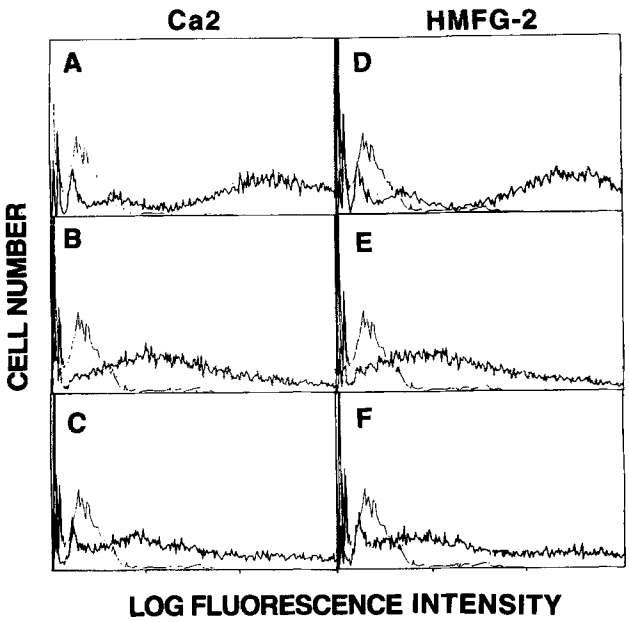
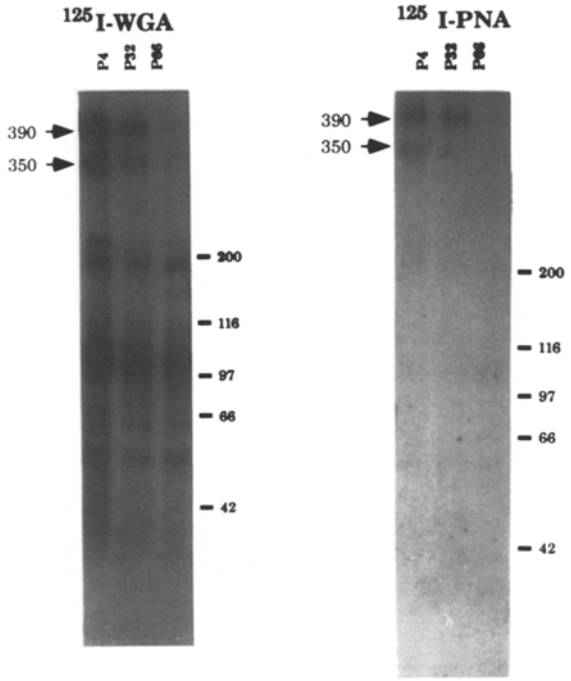


Figure 1. Fluorescence activated cell sorting (FACS) analysis of Ca2 and HMFG-2 binding to H.Ep.2 cells of increasing passage number. H.Ep.2 cells were labelled with the Ca2 or HMFG-2 antibody by indirect immunofluorescent labelling and the binding quantitated by FACS analysis. Antibody binding to cells of passage 3 (A and D), passage 78 (B and E) and passage 114 (C and F) is shown. Non-specific binding (dotted line) and Ca2 (A, B, C) or HMFG-2 (D, E, F) binding (solid line) are shown.



Densitometry (ODxcm)

WGA:

Band	P4	P32	P66
390kD	0.286	0.125	0.027
350kD	0.216	0.090	0.030
100kD	0.457	0.328	0.389

PNA:

Band	P4	P32	P66
390kD	0.140	0.113	0.012
350kD	0.065	0.031	0.022

Figure 2. SDS–PAGE and lectin overlay of H.Ep.2 cell extracts. H.Ep.2 cells of passage 12 (P4), 96 (P32) and 198 (P66) were harvested and extracted as described in the text. The extracts were evaluated by electrophoresis on a 3.5–12.5% acrylamide gradient gel; 25 μg of total cell protein was loaded in each lane as indicated and the glycoproteins were detected by either [^{125}I]WGA or [^{125}I]PNA overlay. Molecular weight marker positions are indicated by the bars, values in kDa. The table delineates the quantitation of band intensities as determined by laser densitometry.

of [^{125}I]lectin bound was quantified by laser densitometry. WGA detected several other bands in addition to the epitectin. The intensity of the epitectin bands decreased 2-fold after 96 passages and a further 4-fold after an additional 102 passages. The densities of the low molecular weight (42–200 kDa) glycoprotein bands were not significantly altered compared with the marked decrease in the density of the epitectin band. This indicates that there are no notable changes in the other WGA-reactive sialoglycoproteins produced by the H.Ep.2 cells [32]. Furthermore, there were no readily observable changes in the Coomassie blue-stained protein bands when equivalent quantities of extracts of the low and high passage cells were examined by SDS–PAGE on gradient gels. PNA bound only the epitectin 350 and 390 kDa material, demonstrating that epitectin is the major glycoprotein of these cells, expressing the disaccharide Galβ1 → 3GalNAcα →, in a form recognizable by the lectin [33]. The PNA-reactive material also decreased with increasing passage number, the 390 kDa band decreased 11-fold between passages 12 and 198, while the 350 kDa band decreased 7-fold (table in Figure 2).

The spent culture medium from the passage 84 cells was found to contain about 6-fold less epitectin compared to medium from passage 9 cells.

Protein epitopes on the H.Ep.2 cell surface

A number of investigators have demonstrated that the monoclonal antibodies Ca1, Ca2 and Ca3 belong to the same family as the monoclonal antibodies generated against human milk fat membranes [11–13]. Whereas the binding of Ca2 antibody to its antigen is dependent on the presence of the intact saccharides [8], the binding of the HMFG-2 antibody to its protein epitope on the mucin glycoprotein does not require the presence of saccharides [34]. We tested the binding of Ca2 and HMFG-2 antibodies to H.Ep.2 cell products by immunoprecipitation of metabolically labelled H.Ep.2 cell extracts. The same [^{3}H]proline labelled 350 and 390 kDa glycoproteins as defined by the lectin overlay were precipitated by both antibodies (Figure 3). Two low molecular weight protein bands (102 and 81 kDa) clearly seen in the HMFG-2 lane are probably the precursor core proteins of epitectin, similar to those observed for other comparable mucin-type glycoproteins [35, 36]. The interactions of the two antibodies with cells treated with either neuraminidase, or a combination of neuraminidase and endo-α-*N*-acetylgalactosaminidase [30], were then analyzed

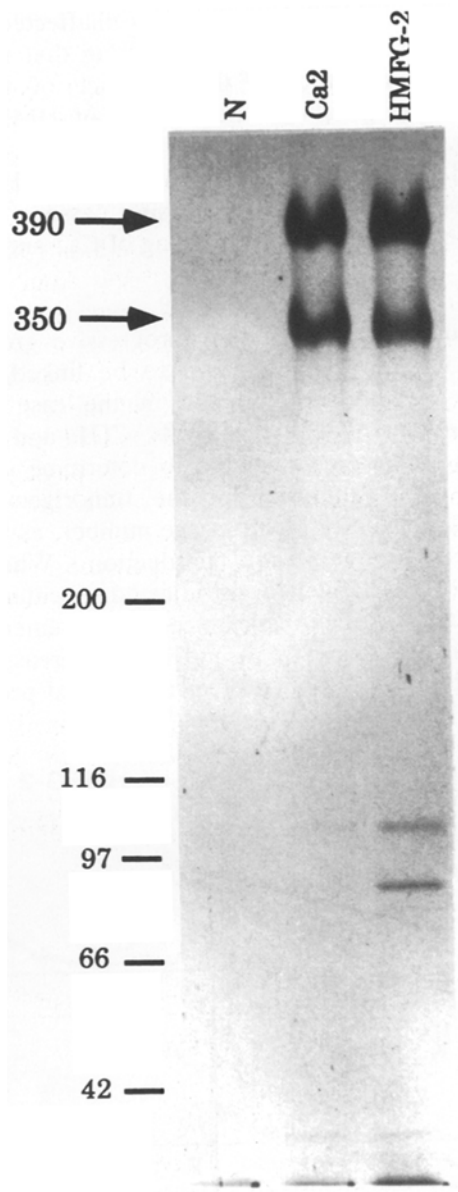


Figure 3. SDS-PAGE of epitectin immunoprecipitated from H.Ep.2 cells labelled with [^{3}H] proline. H.Ep.2 cells which had been labelled with [^{3}H] proline were extracted and immunoprecipitated with a non-specific mouse immunoglobulin (N), the Ca2 monoclonal antibody (Ca2) or the HMFG-2 monoclonal antibody (HMFG-2). The precipitates were evaluated by electrophoresis on a 3.5–12.5% gradient acrylamide gel followed by fluorography. Positions of 390 and 350 kDa epitectin bands and of molecular weight standards are indicated by arrows or bars.

by flow cytometry. The Ca2 antibody binding was increased slightly after desialylation, but decreased one log of fluorescence after the treatment with neuraminidase and endo-α-*N*-acetylgalatosamini-

dase. The HMFG-2 binding was unaffected by the enzyme treatments, thus confirming that its binding does not require the presence of the saccharides (Figure 4). The binding of HMFG-2 to H.Ep.2 cells of varying passage number is illustrated in Figure 1(D–F). The decrease of HMFG-2 antibody binding with increasing passage number paralleled the decreased binding of Ca2 antibody.

Tumorigenicity studies

Malignancy, as defined by progressive growth *in vivo*, was previously shown to be linked to the ability to produce epitectin in the case of the matched pair of hybrid cells CG1 and CGL3 [6, 23]. We were interested to determine whether there is an alteration in the tumorigenicity of H.Ep.2 cells after high passage number, as a result of decreased epitectin production. When 10^6 CGL1 or CGL3 cells were injected subcutaneously in four sites of athymic mice, in agreement with previous findings, we found that whereas CGL3 cells showed a 100% take incidence and produced progressively growing tumors, the non-epitectin producing CGL1 cells did not produce tumors at any site (data not presented). In the case of the low and high passage H.Ep.2 cells, doses of 10^3 to 10^6 cells per site were tested and five separate experiments were carried out. With an inoculum of 10^4 cells per site or lower, neither the low passage nor the high passage cells produced tumors. Typical results obtained with doses of 10^5 and 10^6 cells are illustrated in Figure 5. At the highest dose of 10^6 cells, tumors were produced at 80% of the sites injected with low passage cells and at 75% of the sites injected with high passage cells. There was no noticeable difference in the latent period before the tumor was first detected in the two sets and even though tumors produced by the high passage cells were smaller, the difference was not statistically different (Student's *t*-test). Injection of 10^5 cells produced tumors at 14 out of 16 sites in the case of the low passage cells and only at two out of 16 sites with high passage cells. There were also notable differences in the latency period and size of the tumors produced (Figure 5) even though the statistical significance of the size difference could not be tested due to low tumor take with high passage cells. The results indicate a link between the tumorigenicity of H.Ep.2 cells in nude mice and the level of epitectin production by these cells.

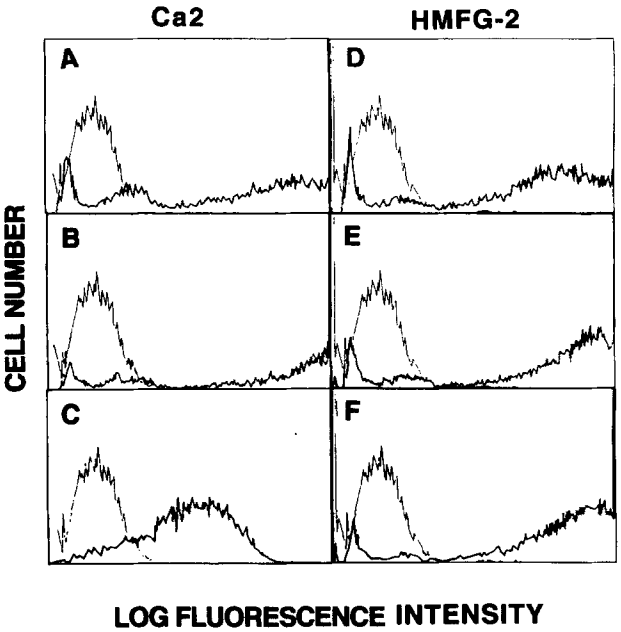


Figure 4. FACS analysis of Ca2 and HMFG-2 binding to H.Ep.2 cells before and after treatment with glycosidases. H.Ep.2 cells were labelled with the Ca2 or HMFG-2 antibodies by indirect immunofluorescent labelling and the binding quantitated by FACS. A, B and C illustrate Ca2 binding; D, E and F illustrate HMFG-2 binding. The dotted line is the non-specific binding, the solid line represents specific binding. A and D, binding to untreated H.Ep.2 cells; B and E, cells treated with neuraminidase; C and F, cells treated with neuraminidase and endo-α-*N*-acetyl galactosaminidase.

Discussion

In this investigation, we report the effect of long-term culture on the expression of epitectin production by the H.Ep.2 cell line. We observed a striking decrease in the level of epitectin detected on the cell surface of H.Ep.2 cells after 78 or more generations when using the Ca2 antibody, WGA or PNA as probes. In contrast, there were no alterations in the Coomassie blue stainable proteins or glycoproteins detectable after SDS–PAGE. Furthermore, we did not observe any significant differences in the doubling time or morphology of the low and high passage cells. While the binding of all three probes for epitectin decreased with increasing passage of cells in culture, the quantitative variations differed between the probes. This is expected since these are known to bind different epitopes. The lectins, WGA and PNA, recognize specific saccharide structures whose spatial arrangement on the core protein may influence binding. Thus, a decrease in WGA binding could be due to a redistribution of the

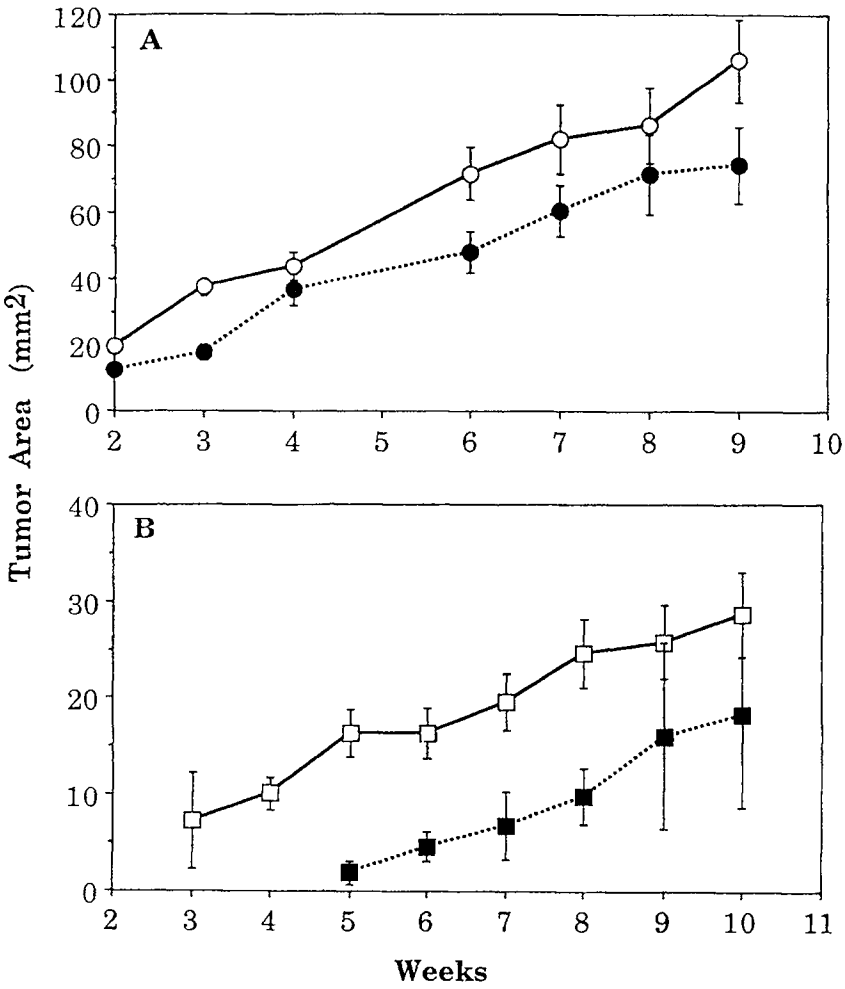


Figure 5. H.Ep.2 tumor growth in athymic mice. A, 10^6 H.Ep.2 cells of passage 12 (○) or passage 108 (●) were injected subcutaneously in athymic mice. Tumor growth was monitored weekly; mean geometric diameter was calculated as described in Materials and methods. Results are averaged for 18 tumors for passage 12 cells and 20 tumors for passage 108 cells. B, 10^5 H.Ep.2 cells of passage 9 (□) or passage 90 (■) were injected and growth followed as above. Results are averaged for 14 tumors for passage 9 cells and two tumors for passage 90 cells. Bars = SE.

same number of sialyl oligosaccharides from a clustered arrangement in low passage cell epitectin to an even distribution in high passage cell epitectin [32]. Since binding of Ca2 antibody is also influenced by glycosylation [8; and Figure 4] it could be that the observed phenomenon is due to aberration in epitectin glycosylation with increasing passage number. However, our results with HMFG-2 antibody argue against such a possibility. Enzymatic deglycosylation, which altered binding of Ca2 antibody to H.Ep.2 cells, did not influence the binding of HMFG-2 (Figure 4), confirming that the latter recognizes a peptide epitope [34]. We have deliberately used very mild conditions for enzyme digestion in order to prevent cell lysis; these conditions were found to cleave substantial *O*-linked saccharides from labelled epitectin in other studies (K. Yamakami and V. P. Bhavanandan, unpublished results). The parallel decrease of both Ca2 and HMFG-2 antibodies to H.Ep.2 cells (Figure 1) clearly demonstrates the loss of cell surface expression of epitectin molecules with prolonged passage in culture. The data shown in Figure 2, which demonstrate the loss of total cellular epitectin as opposed to cell surface epitectin as detected by flow cytometry, excludes the possibility that the high passage cells are synthesizing similar levels of epitectin which are not transported to the cell surface. Since the media from high passage cells also had less epitectin than media from low passage cells, the decrease of cell surface epitectin is not due to increased secretion/shedding by the former cells. We do not know at this time if the decrease in epitectin expression is due to decreased transcription or translation, or a processing malfunction of the cells. Alternatively, the decrease could be attributed to a dedifferentiation of the cells; perhaps the tissue culture environment lacks some stimulus necessary for the cells to produce epitectin. Bader and Harris have noted that lactate induced increased epitectin production by the RT112 cell line (derived from human bladder carcinoma), demonstrating the inducibility of epitectin under conditions of hyperos-

molarity (37). It has been reported that two other malignancy-associated mucin glycoproteins [epiglycanin and ascites sialoglycoprotein-1 (ASGP-1)] are produced by TA_3 Ha mouse and the 13762 rat mammary adenocarcinoma cells, respectively, only when the cells are grown in the ascites form [38, 39]. Growth of these cells in culture resulted in the loss of mucin expression. However, there was no loss of tumorigenicity and the loss of mucin was reversible. Furthermore, the cells regained production of these glycoproteins when they were passaged in the ascites form [38, 40].

Since the H.Ep.2 cells received from ATCC had already been cultured fairly extensively, the reason for the noted decrease in epitectin production as a result of continued cultivation *in vitro* is not clear. It is likely that differences in culture conditions, including the source and exact composition of media and/or serum, could be contributory factors. One notable difference from previously is that we have avoided the use of trypsin during subculturing and harvesting of cells, to prevent loss of cell surface epitectin. In contrast, the ATCC procedure involves treatment with 0.25% (w/v) trypsin for 1–2 min. Whatever the reason for the observed phenomenon, the investigation of the low and high passage H.Ep.2 cells should be useful for investigating the relevance of epitectin production in malignancy.

Epitectin was originally discovered after noting the ability of the Ca1 antibody to distinguish between malignant and non-malignant segregates of hybrid cells which were derived from cervical carcinoma and diploid fibroblast cell fusions [6]. In the case of CGL 1 and CGL 3 cells, the ability to produce epitectin appears to be linked to the ability of the cells to grow progressively *in vivo*. Analogously, we sought to compare the tumorigenicity of H.Ep.2 cells of varying epitectin production levels in nude mice. Following subcutaneous injection, tumor growth was measured weekly and tumor area calculated. With an inoculum of 10^5 cells per injection site, a clear correlation existed between tumorigenicity and the level of epitectin synthesized by the H.Ep.2 cells. At the higher inoculum of 10^6 cells there was no significant difference in tumorigenicity between the high and low passage cells. This is not unexpected since even though there is an overall decrease in epitectin production by the high passage cells, this cell population consists of a broad spectrum of epitectin producing cells (Figure 1). Thus, it can be expected that there are sufficient high epitectin producing cells in the 10^6 cell inoculum to produce tumors. These observations are further evidence for the link between the ability of human cells to express epitectin at the cell surface and their ability to produce tumors in nude mice. In this study, we could not test any role epitectin may play in the immunology of the tumor, because it was necessary to use immunosuppressed mice for efficient tumor growth. Future studies will address the regulation of epitectin production in the cultured H.Ep.2 cell lines.

Acknowledgements

We are grateful for the support and help by the personnel (Dr B. M. Ohlsson-Wilhelm, Mr Nate Sheaffer and Ms Tammy Hyde) of the Flow Cytometric Facility at this Institution. We thank Prof Henry Harris and Dr Mike Bramwell, Oxford University for supplying the Ca2 antibody used in these studies and Professor Eric Stanbridge, University of California, Irvine, for providing the CGL1 and CGL3 cell lines. We also thank Ms Shani Williams for assistance with ELISA, and Ms Fil Cramer for secretarial assistance. This investigation was supported by US PHS grant CA38797 awarded by the National Cancer Institute.

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(Received 12 October 1993; accepted in revised form 5 January 1994)