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BINDING OF EPIDERMAL GROWTH FACTOR BY HUMAN COLON CARCINOMA CELL (CACO-2) MONOLAYERS

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SUMMARY: The objective of this study was to investigate whether Caco-2 cells bind and internalize epidermal growth factor (EGF). [^{125}I]EGF was presented to the apical (AP) or basolateral (BL) side of Caco-2 monolayers, grown on microporous membranes, at different times in culture. At day 10, [^{125}I]EGF binding (at 37 °C) to the BL membrane was 2-3 times greater than binding to the AP membrane. Of that [^{125}I]EGF bound to the AP membrane 76 % was internalized within 3 h while internalization from the BL membrane was 90 %. At lower temperatures membrane-bound [^{125}I]EGF increased while internalization decreased. At day 16, AP and BL binding decreased and then remained constant through day 25. [^{125}I]EGF was bound to the BL membrane of 10 days old monolayers with a K_d of 0.67 nM. There was a single binding site whose numbers in the BL membrane was about 5500/cell.

Epidermal growth factor (EGF) is a 53 amino acid polypeptide (MW 6045) with a wide range of biological effects, such as enhanced protein and DNA synthesis, increased cell proliferation and modulation of cellular pH and ion transport (1,2). Although the exact mechanism by which EGF exerts its physiological role is not clearly understood, recent studies support the trophic effects of orally administered EGF on newborn and adult rat small intestine (3,4). There is also evidence that EGF binding to receptors located in enterocyte membranes is followed by internalization of the receptor-ligand complex and triggering of some of the biological responses associated with this peptide (5).

The presence of EGF in maternal milk of humans and other mammalian species (6,7) and the trophic effect of colostrum on suckling animal intestine appear to substantiate the regulatory role of EGF in enterocyte proliferation (8).

Several reports have demonstrated the presence of EGF receptors on intestinal epithelial cells (9-12) and that EGF undergoes transmucosal transport across suckling rat intestine

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(13). However, until very recently, there has been a lack of information on the polarity of the EGF receptor in the small intestinal epithelium (14).

Recent studies have demonstrated that the human colon carcinoma cell line, Caco-2, undergoes enterocytic differentiation in culture (15). Subsequently, we have shown that Caco-2 cells grown on a microporous support may represent a valuable model of the small intestinal epithelium (16). The objective of this study was to determine whether the Caco-2 cell monolayer may be a useful tool in furthering our understanding of the biological interactions of EGF with the small intestinal epithelium. Using Caco-2 cell monolayers, we demonstrated the presence of EGF receptors in Caco-2 cells. We also examined the effect of temperature and time in culture on EGF binding. Finally, quantitative characterization of EGF receptors enabled us to determine the distribution of EGF receptors between apical (AP) and basolateral (BL) membranes, the binding affinity and the number of binding sites present on Caco-2 cells.

MATERIALS AND METHODS

Materials

The Caco-2 cell line was obtained from American Type Culture Collection, Rockville, MD, and was used between passages 65 and 80. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and non-essential amino-acids (NEAA) were obtained from Hazleton Research, Lenexa, KS. Transwell™ clusters , PVP free, 24.5 mm diameter (4.71 cm^2 surface area) and 3.0 mm pore size were purchased from Costar, Bedford, MA. Rat tail collagen (Type I) was purchased from Collaborative Research, Lexington, MA. EGF (receptor grade) was obtained from Calbiochem, La Jolla, CA and [^{125}I]EGF (90-140 mCi/mg) from ICN Radiochemicals, Irvine, CA. All other reagents were reagent grade.

Cell Culture

Caco-2 cells were plated at a density of 63000 cells/cm^2 on Transwell™ polycarbonate membranes, which had been coated with collagen as per supplier's instructions. The culture medium, consisted of DMEM, 10% FBS, 1% NEAA and 1% L-glutamine. The culture medium was replaced (1.5 ml inside and 2.6 ml outside) daily. Cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity.

Binding studies

Caco-2 cell monolayers were washed three times with serum-free DMEM. [^{125}I]EGF binding was determined by applying 2 ml binding medium (DMEM + 0.1% BSA) that contained 0.5 or 1.0 ng/ml [^{125}I]EGF to the AP or BL side while the side opposite to that in which [^{125}I]EGF was applied contained only binding medium (BM). Nonspecific binding (NSB) was determined in the presence of a 50-fold excess unlabeled EGF and specific binding was obtained by subtracting NSB from total binding at each time point. Subsequently, the monolayers were incubated at either 37, 23 or 4 °C for specified (indicated on the appropriate figure legend) lengths of time. At the end of the incubation period, the

monolayers were washed three times with ice-cold BM. Membrane-bound [^{125}I]EGF was stripped by acidifying with ice-cold 1 M glycine buffer, pH 3.0 for 2 min. Since this acidification technique removes only peptide ligands bound to receptors on the cell membrane (17), that bound [^{125}I]EGF that was not removed by this treatment was assumed to have been internalized. Intracellular [^{125}I]EGF was determined by harvesting the cells with 1 ml 0.01% Triton-X and then counting the samples for 5 min in a Beckman DP5500 Gamma Counter. Protein was determined by the method of Lowry et al [18]. The nonlinear regression analysis program LIGAND (19) was used to estimate the binding parameters K_d (dissociation constant) and B_{max} (maximum binding capacity).

RESULTS

Both AP and BL binding of [^{125}I]EGF decreased from day 10 to day 16 but no additional reduction was seen at day 23 in culture (Figure 1). Despite this decrease in binding, the fraction of [^{125}I]EGF specifically bound to Caco-2 cells that was internalized did not change with time in culture. The amount of [^{125}I]EGF bound to the BL membrane was 2 to 3 times higher than that bound to the AP surface (Figure 1). In addition, 2 h after BL administration of [^{125}I]EGF 85 % of that [^{125}I]EGF associated with the monolayer was internalized. In contrast, after AP administration the fraction of [^{125}I]EGF bound to the cell membrane in 2 h was approximately the same as that which was internalized.

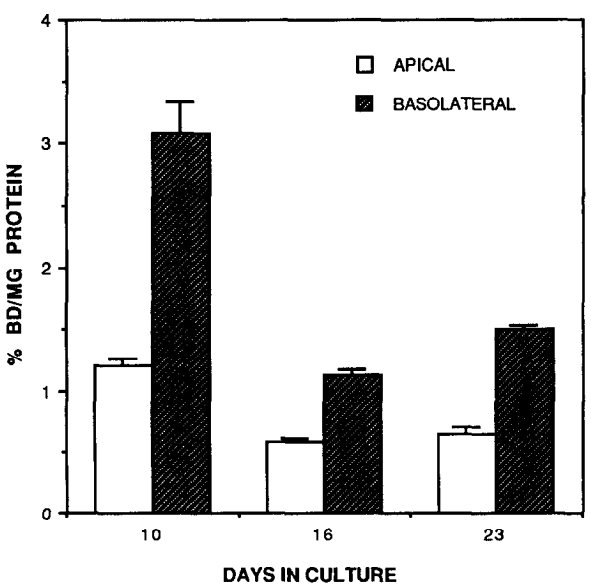

FIGURE 1. Effect of time in culture on AP and BL binding of [^{125}I]EGF. Caco-2 monolayers were washed and then incubated with 2 ml of 1.0 ng/ml [^{125}I]EGF on the AP or BL membrane for 3 h at 37 °C. At the end of the incubation, the monolayers were treated as indicated in Materials and Methods. Values shown represent specific binding of three monolayers ± SD. 100%=0.33 pmol. Key: open bars, AP binding; hatched bars, BL binding.

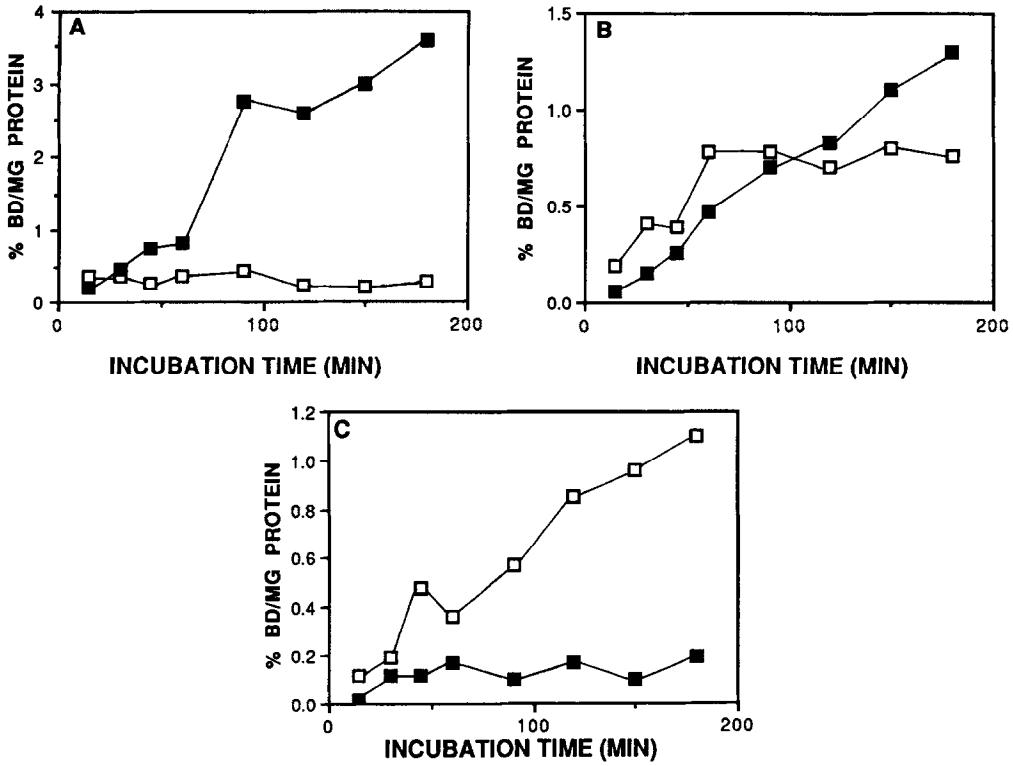

FIGURE 2. Temperature-dependence of [^{125}I]EGF binding and internalization. 10 days old Caco-2 cell monolayers (N=3) were incubated with 0.5 ng/ml [^{125}I]EGF, on the BL side, for 3 h at 37 ° (**A**), 23 ° (**B**) or 4 °C (**C**). Open squares, membrane-bound; black squares, internalized. Errors were (normally less than 15 %) were omitted for clarity of presentation.

Binding experiments indicated that the number of EGF receptors on Caco-2 cells was small. Also, the BL-to-AP [^{125}I]EGF binding ratio was 2-3, indicating that EGF receptors are more abundant in the BL membrane than in the AP domain. Thus, in additional experiments [^{125}I]EGF was applied only to the BL surface, and not to the AP membrane, of 10 days old (when [^{125}I]EGF binding was higher) monolayers.

The binding and internalization of [^{125}I]EGF by the BL membrane was further investigated in 10 days old Caco-2 cell monolayers. Here, a decrease in temperature caused more [^{125}I]EGF to remain at the surface ($P<0.05$) and less to be internalized (Figure 2).

[^{125}I]EGF was bound with high affinity as indicated by a K_d of 0.67 nM. [^{125}I]EGF binding was competed efficiently by unlabeled EGF (Figure 3) and Scatchard analysis indicated the existence of a single binding site (Figure 3, inset). When the concentration of unlabeled EGF was 100-fold excess, [^{125}I]EGF internalization was completely abolished (Table I). Using the estimated B_{max} (22 fmol/mg cellular

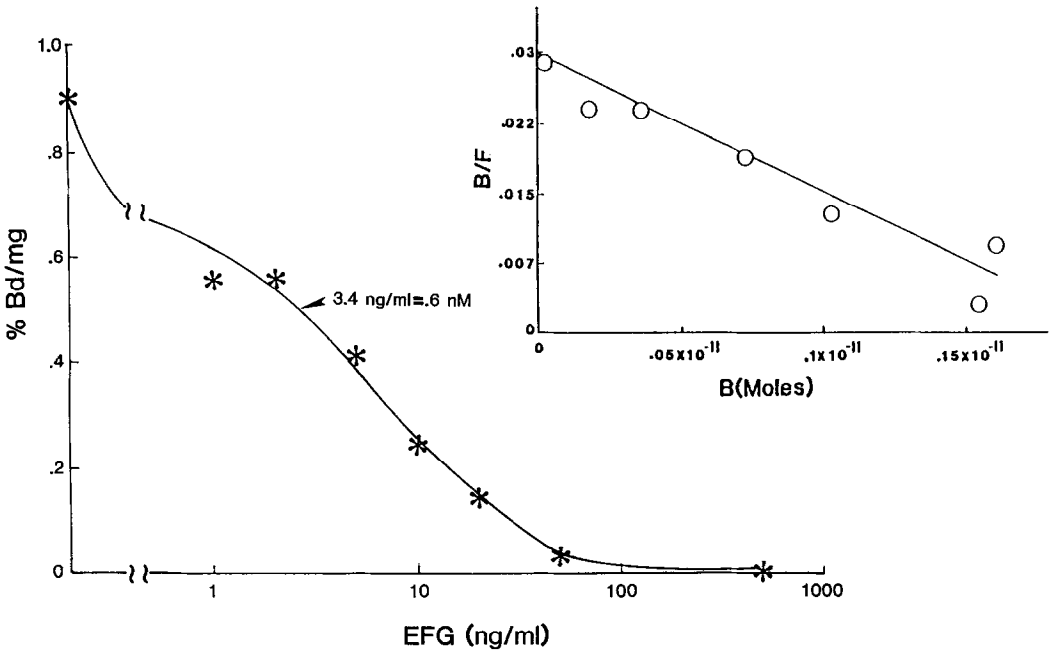

FIGURE 3. Displacement of [^{125}I]EGF by unlabeled EGF. Cell monolayers were incubated with 0.5 ng/ml [^{125}I]EGF, on BL side, alone and in the presence of 1, 2, 5, 10, 20, 50 and 500 ng/ml unlabeled EGF for 3 h at 23 °C. The results have been corrected for non-specific binding. Each point is the average of three monolayers. The inset shows a Scatchard plot of the data.

protein) and the number of cells per mg protein (1.6 x 10^6) the number of receptors on the BL membrane of Caco-2 cells was calculated to be approximately 5500 receptors/cell.

EGF inhibited virtually all binding and internalization of [^{125}I]EGF (Table I). [^{125}I]EGF internalization was inhibited by Na

TABLE I

EFFECT OF EXPERIMENTAL CONDITIONS ON [^{125}I]EGF BINDING AND INTERNALIZATION[a]

Treatment	Membrane-Bound[b]	Internalized[b]
-	0.50 ± 0.05 (100)[c]	3.33 ± 0.25[c] (100)
NaF+NaN_3[d]	0.25 ± 0.03 (50)*,[f]	0.14 ± 0.001 (4.2)**
EGF[e]	0.01 ± 0.003 (2)**	0.03 ± 0.006 (0.9)**
4 °C	0.32 ± 0.03 (64)*	0.05 ± 0.006 (1.5)**

[a] Monolayers were incubated for 2 h at 37 °C either with 1.0 ng/ml [^{125}I] EGF alone (control) or as indicated.
[b] % Bound/mg protein
[c] Values are mean ± SD (N=3-4) and numbers in parenthesis are % of control
[d] NaF, 20 mM; NaN_3, 5 mM
[e] 50 ng/ml
[f] *, $P < 0.05$; **, $P < 0.01$ compared to controls (ANOVA and multiple range t-test).

azide ($P<0.001$) and at 4 °C ($P<0.001$) (Table I) both of which had also a significant ($P< 0.05$) although less pronounced effect on binding (Table I).

DISCUSSION

Since it has been demonstrated that the expression of marker enzymes and specific carriers in Caco-2 cells reaches a maximum after the cells have formed a complete monolayer of polarized cells (15,16), binding studies were carried out on cell monolayers that had been cultured for different lengths of time. Our results appear to indicate that EGF receptors, are more numerous at early times, and decrease when the cells become more differentiated. This observation is consistent with a previous study showing that EGF binding in isolated human intestinal and colonic cells from 17 weeks old fetuses was 50% lower than in 12 weeks old fetuses (20). A decrease in EGF binding along the crypt-villus axis (11), suggests a loss of receptors with increasing cell differentiation.

The K_d of EGF binding in Caco-2 cells (0.67 nM) is comparable to those reported for fetal (1.03 nM) and adult (2.31 nM) rat intestinal microvillus membranes (12).

Caco-2 cells exhibit substantial polarity with respect to marker enzymes (15,16), the transport of bile acids (21) and neutral amino acids (21) and the secretion of lipoproteins (22). In contrast the distribution of EGF receptors between AP and BL membranes is consistent with partial polarization. While the BL-to-AP ratio of EGF receptors is 2.5:1, the corresponding ratio of human transferrin receptors (hTfRs) was 14:1 at day 14 and 8:1 at day 23 (23), indicating that the sorting of hTfRs is much more accurate than that of EGF receptors. The polarities of both hTfRs and of EGF receptors in Caco-2 cells are low relative to other epithelial cell lines. A recent study showed that filter-grown Madine-Darby canine kidney (MDCK) cells displayed BL-to-AP ratios for TfR of 800:1 and 300:1 for the high and low resistance strains, respectively (24).

Proportionately more of that EGF bound to the BL membrane was internalized relative to the AP counterpart. The higher EGF binding by the BL membrane may be explained by a higher density of receptors or, more likely, by the larger surface area of this domain. For example, von Bonsdorff et al (25) found that in MDCK cells an apparent disparity in pinocytic uptake rate that favored the BL domain is due to differences in membrane surface area (in MDCK cells the BL membrane is 7 times larger than the AP membrane). The experiments in which EGF was only applied to the BL membrane of 10 days old monolayers provided useful information on the the EGF receptor. The internalization of

EGF was more susceptible to inhibition by Na azide and 4 °C, and unlabeled EGF inhibited both [^{125}I]EGF binding and internalization completely, indicating receptor-mediated uptake.

This study shows that the Caco-2 cell monolayer system can be useful for investigating the binding and endocytosis of EGF. As Caco-2 cells resemble the enterocytes closely, they may be useful to study the manner in which EGF exerts its numerous effects on the small intestinal epithelium as well as its potential transepithelial transport. The proposed role for EGF in intestinal development (1,2,7) may explain their presence on the BL membrane of Caco-2 cells, but a physiological role for EGF on the AP membrane of enterocytes remains unclear.

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