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The use of Caco-2 cells as an *in vitro* method to study bioavailability of iron

Maznah Ismail

Department of Nutrition and Health Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

ABSTRACT

Iron absorption is essential for the maintenance of iron levels in the body, since excretion is poorly regulated. Dietary factors can influence iron absorption including low molecular weight substances such as ascorbic acid which has been shown to enhance iron transport across mucosal cell monolayers. Both *in vivo* and *in vitro* work may be carried out to study iron absorption. Studies *in vivo* have the drawback of dealing with a complex system in which it is difficult to determine the relative importance of different factors. In *vitro* cell culture models could overcome this difficulty but attempts to establish differentiated enterocyte cell lines in culture have not been successful. However the Caco-2 line, derived from a colon carcinoma, is able to differentiate spontaneously when grown in standard culture conditions. The differentiated cells polarized, formed microvilli and T-junctions associated with the duodenal enterocytes brush border. This cell line thus represents an appropriate model for the study of transport mechanisms related to the intestinal barrier and can be used to study the absorption of nutrients especially iron in relation to dietary intake in particular pertaining to dietary factors that may affect absorption. In this work we have therefore used differentiated Caco-2 cells grown in bicameral chambers as a intestinal cell model to study the absorption of iron from different sources and compared it with INT 407 cells. Transfer of iron across the monolayers in the apical-to-basolateral direction has been found to be greater from feric lactoferrin than from iron citrate, while very little transport occurred from Fe-transferrin. It is concluded that in this in vitro study lactoferrin but not transferrin enhances mucosal iron transport. More importanty this study has also shown that Caco-2 can be used as an *in vitro* method to investigate not only iron bioavailability but can be applied to other minerals as well.

INTRODUCTION

Bioavailability refers to the proportion of an ingested nutrient from foods or meals that is absorbed and utilised for normal physiological function and/or storage (Jackson, 1997). It is affected by various physiological parameters in the animal and human that consumes the food, but more important from the practical point of view, bioavailability of many nutrients is modulated by other dietary components. These dietary components may be an organic compound that binds the nutrients or another metal which interacts with it. Prior treatment of food and food products such as in cooking and processing may also affect bioavailability of nutrients.

Apart from dietary factors, physiological factors unrelated to the properties of the foodstuffs are also likely to influence the properties of a nutrient being absorbed. These include such factors as the efficiency of digestion, the previous intake of the nutrient, gut transit time and the presence of gastro-intestinal disorder or disease. Nutritional status of an individual is of paramount importance in determining bioavailability as absorption often parallels to the individual's needs. In general, absorption is increased in deficiency and depressed in overload.

From practical view point, determining the bioavailability of a mineral is complicated by the fact that once a food is consumed, it mixes in the gastro-intestinal tract with other foods that are consumed at about the same time or may be because the minerals are present in a mixture of sources available in the diet or meal. Furthermore minerals contained in a meal may also mix with the remains of the previous meal. The use of extrinsic radioiron tagging and stable isotopes in humans has partly offers a solution to this problem. Apart from the advantage of using an intact biological system, it has greatly advanced our knowledge on factors affecting the absorption of minerals especially that of iron. However these measurements are cumbersome, time consuming and costly to perform. Furthermore it is difficult to determine the relative importance of different factors if environmental conditions are not controlled properly.

These limitations have restricted the use of mineral absorption studies in human for the purpose of identifying specific biochemical components of the diet that explain the inhibitory or enhancing nature of certain foods. The measurements would be greatly facililated by the development of a simple *in vitro* screening method to assist in characterising the biochemical basis of food iron bioavailability. In addition, understanding metabolic pathways of minerals as affected by dietary factors could be further elucidated in order to formulate diets.

In vitro methods have been used to estimate mineral bioavailability for at least 50 years. Three approaches with various modifications had been used, the first being measurement of "ionizable" or "ionic" iron in foods. This is done by determining the function of the total iron in a food that will react with a complexing agent such as dipyridyl and then

quantitated spectrophotometrically . The second approach is to subject the foods to a stimulated gastric and gastrointestinal digestion using purified peptic and/or pancreatic enzymes with subsequent measurements of the soluble iron released by the digestion (Miller *et al.* 1981). More current approach in the *in vitro* study of mineral bioavailability is the use of brush border membrane vesicles and cultured epithelial cells. However, attempts to establish differentiated enterocytic cell lines in culture have not been successful as, though expressing several brush border enzymes, these cells lack the structural characteristics of differentiated enterocytes (Grasset *et al.*, 1984).

However, Caco-2 line derived from a colon carcinoma is able to differentiate spontaneously when grown in strandard culture conditions (Sanchez, *et al.,* 1996). The differentiated cells polarise, form microvilli and secrete enzymes associated with the duodenal enterocyte brush border such as sucrase-isomaltase, alkaline phosphatase, alkaline phosphatase, lactase and aminopeptidase (Rousset, 1986). This cell line thus represents an appropriate model for the study of transport mechanisms related to the intestinal barrier and for investigating diverse problems of nutrients bioavailability and absorption without concern for differences between human and animals. This study was therefore conducted in order to establish the use of Caco-2 in comparison to INT 407, an ileal jejunal cell line as a model for in vitro bioavailability determination specifically for the use in understanding the transport of iron from different sources, namely ferrous citrate, lactoferrin and transferrin.

MATERIALS AND METHOD

Cells

Caco-2 cells, colon carcinoma cell line and INT 407 cells were obtained from American Type Culture Collection (ATCC), USA.

Cell Cultivation

Caco-2 cell were routinely cultured in 25 cm^2 tissue culture flask (Costar, UK) with Dulbecco's modified Eagle medium (DMEM) (Gibco, Paisley,UK) supplemented with 10% fetal calf serum (Northumbria, Cramlington, UK), I% non-essential amino acids (Flow, Rickmansworth,UK), I μg/ml bovine insulin (Sigma, Dorset, UK) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin (Flow) at 37 °C with 5% CO_2. INT 407 cells were cultivated for routine culture in RPMI medium (Flow, UK) without HEPES with similar supplementations. Cells were sub-cultured with 5 ml EDTA (1 mM, BDH,UK) and 5 ml trypsin (0.25% Sigma) for every 6-7 days.

Transwell Bicameral Chambers

For bioavailability studies, cells need to be cultured in Transwell Bicameral Chamber (Costar, High Wycombe, UK) (Fig 1) until they form complete monolayers. Inside each Transwell bicameral chamber is an insert with the inside wall being treated for uniform cell attachment. The surface area of the membrane is 0.33 cm^2 with o.3 μm pore size and

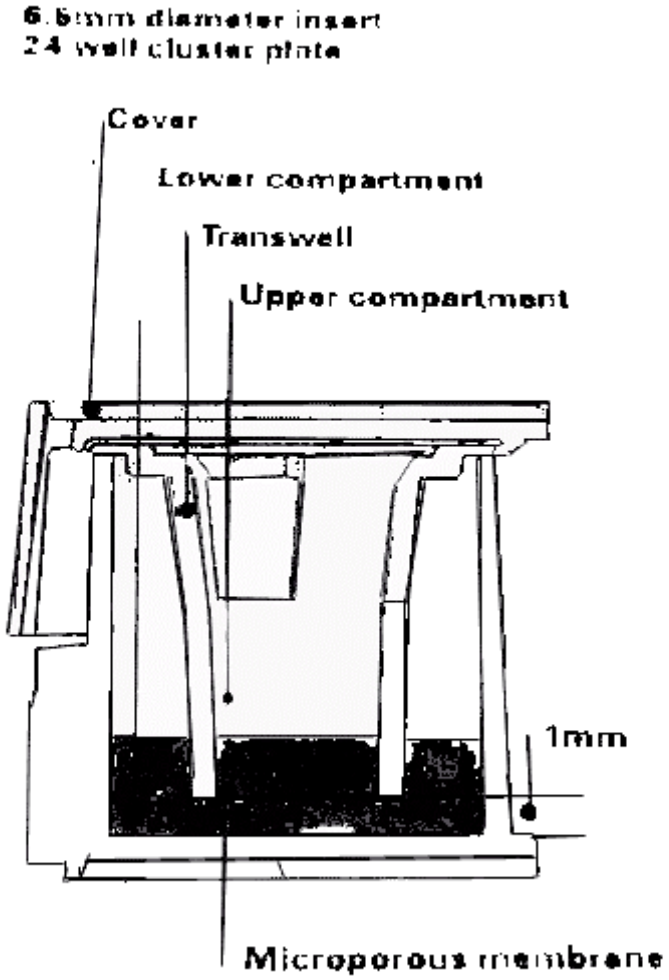

Figure 1: Transwell Bicameral Chambers

6.5 mm diameter size. The transwell bicameral chamber has an upper and lower chamber.

Cell Cultivation for Transport Studies

When confluent, cells were seeded into transwell bicameral chamber at a density of 10^5 cells/cm^2. The polycarbonate membrane of the inserts was previously precoated with collagen by adding 50 µl of a 2 mg/ml solution of rat tail collagen type 1 (Boehringer Mannheim, Germany) in 0.1 M acetic acid to each chamber. Excess solution was removed and the inserts were

dried inverted under sterile conditions. The cells were maintained at 37 °C in an atmosphere of 5 % CO_2 and 95% relative humidity and the medium changed daily. Confluent cultures of differentiated cells were obtained after 17-20 days by measurement of transepithelial electrical resistance (TEER) with epithelial voltohmeter (World Precision Instrument, New Haven, CT USA). The instrument has a chopstick like electrodes with the short electrode being inserted in the Transwell insert whereas the longer electrode is placed in the lower chamber. Electrical resistance values of cell monolayers in the inserts were measured daily and values were multiplied by surface area minus the resistance of blanks of insert without cells.

Phenol red exclusion was used as a measure of monolayer integrity. In brief, the culture medium in the lower chamber was removed and the cells were washed with 2 x 500 µl phenol red free HBSS after which the lower chamber was added 800 µl phenol red free HBSS. The Transwell insert was later placed in the lower chamber and diffusion of phenol red was allowed at 37 °C for 2 1/2 h in the incubator. Aliquots of 100 µl were removed at time intervals from the upper and lower chambers and A_{546} nm were determined and percent diffusion was calculated.

Transmission Electron Microscopy

Caco-2 cells grown in Transwell chambers were examined by transmission electron microscopy using standard procedures. Briefly, cells were fixed overnight with 2 % glutaryldehyde followed by post-fixation in 1% OsO_4 and embedding in Araldite. Sections (2 µm) were stained with toluidine blue and examined by light microscopy. Ultrathin (90 nm) sections were then cut from suitable areas, mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a philips CM10 electron microscope.

Iron Transport Studies

Caco-2 monolayers grown in bicameral chambers were used for transport studies when they had differentiated and the monolayer intact, as checked by measuring TEER and phenol red exclusion. After washing both the upper and lower chambers with serum-free medium, lactoferrin and transferrin solutions were added to the upper chambers with serum-free medium , lactoferrin or transferrin solutions were added to the upper chamber. Both proteins were 50% saturated with [^{59}Fe]- citrate (specify activity 10 *u*g, Amersham) and added at a concentration of 50 *u*g / ml. Saturation to 100% was then achieved using unlabelled ferric nitrilotriacetate (FeNTA) as described previously [23]. This procedure ensures that proteins are fully saturated , but avoids of any free ^{59}Fe being present. Transport of iron from Fe-citrate was assesed by adding an equivalent amount of [^{59}Fe]-citrate to the cultures in the absence of transferrin or lactoferrin. The final concentration of ^{59}Fe in the samples was 470 nM. The lower chamber contained human apo-transferrin (1 mg / ml) as iron acceptor. Serum - free medium was used during iron uptake and transport in order to ensure that total iron levels in the medium could be controlled. Use of serum-free

medium did not affect the TEER. Medium from the lower compartment was removed for analysis at 1, 5 and 23h of incubation and replaced by fresh medium. At the end of the experiment, the TEER was measured, the cells were washed three times with Hank's solution and were then dissolved in 2% (w/v) SDS. Radioactivity associated with the medium from the upper and lower chambers at different times of incubation, and with the SDS-digest of the cells was determined.

RESULTS

Transepithelial Electrical Resistance

TEER values across the collagen-treated polycarbonated membrane without cells ranged between 35-49 Ωcm^2 (Figure 2). Tests on uncoated membranes indicated that the electrical resistance due to collagen layer was only 6-8 Ωcm^2. For the first four days after seeding the cells on to the polycarbonated membrane, the TEER values of both cell monolayers were only slightly higher than the background, about 80 Ωcm^2. As for Caco-2 cells, from day 5 the TEER values increased gradually until reaching maximum (265 Ωcm^2) on day 17 followed by constant readings afterwards. With INT 407, observations under the light microscope showed a good monolayer was formed by day 7, however these cells did not show any increment of TEER values with time and they were only slightly higher than the background values, 78.1 Ωcm^2 (Fig.2).

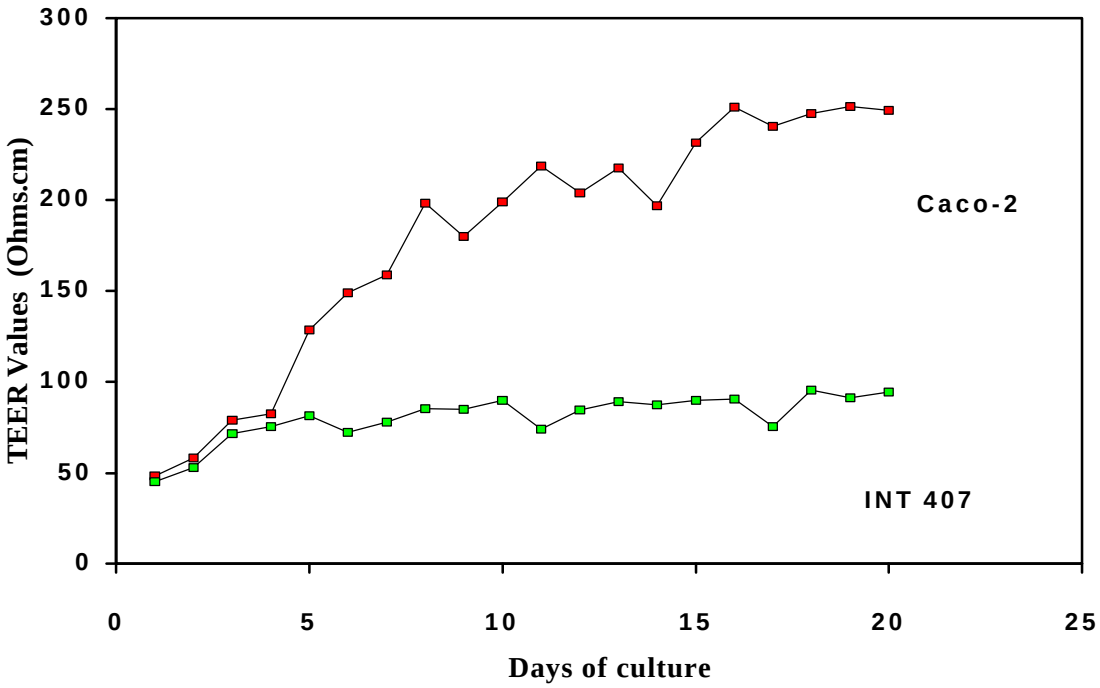

Figure 2: Transeptihelial electrical resistance (TEER) values of Caco-2 cell monolayers cultured in Transwell bicameral chambers. Each point represents a mean of at least 4 inserts.

The TEER values were also measured before and after every transport experiment. These values were found to be unchanged after the experimentation, suggesting that the monolayers remained intact during and after the transport studies.

Phenol Red Exclusion

Passage of phenol red already present in the culture medium from the upper compartment to the lower compartmnet, filled during the assay with phenol-red free medium traversing the cell monolayer was used as another simple and non-destructive test to determine the confluency of the cell monolayers. After two days of culture the percentage diffusion of phenol red from the upper to lower chambers across the cell monolayers was about 15% (Figure 3). This value was close to the background in the absence of cells (17%). The values decreased gradually for Caco-2 until reaching a constant on day 17 which is consistent with the TEER readings. As for INT 407, the decrease in percent diffusion is much faster than Caco-2, indicating that INT 407 formed confluent monolayer earlier than Caco-2 cells. However, results from TEER indicated that the monolayer was not polarised. Similar readings of percent phenol red diffusion taken before and after each individual experiment remained the same in support of the TEER readings.

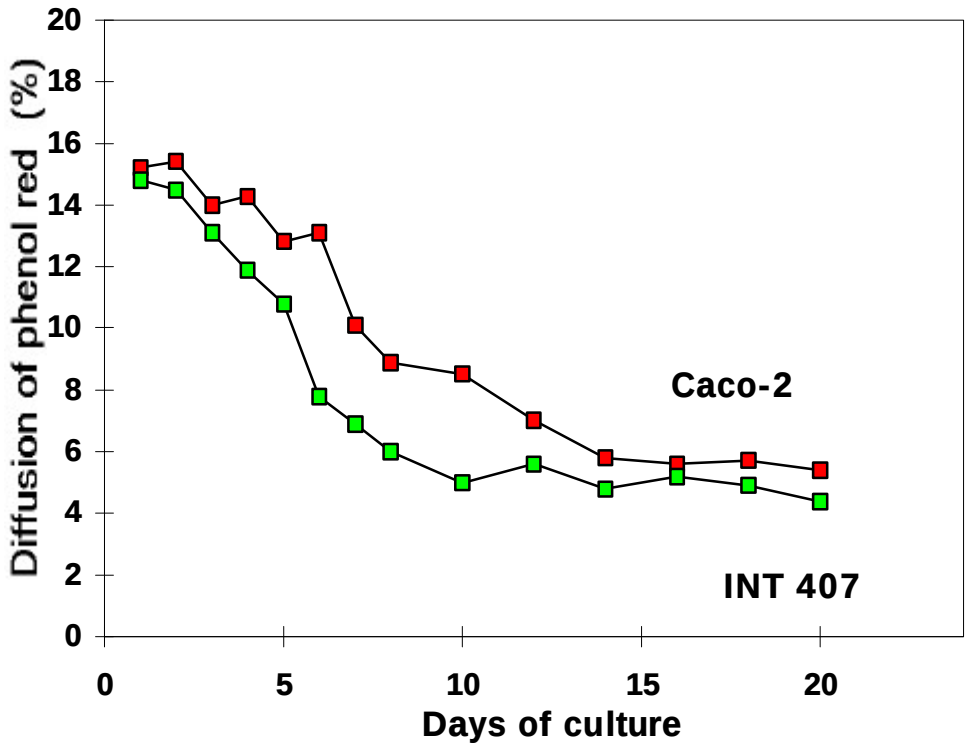

Figure 3. Passage of phenol red across Caco-2 cell monolayers cultured in Transwell bicameral chambers.

Cell Morphology

The degree of morphologic differentiation of Caco-2 cells with the expression of microvilli and tight junctions and the ability of the cells to form complete monolayers on collagen coated, polycarbonate membrane were assessesed by examining the cell morphology after day 17. Figures 4 and 5 show that the cells demonstrated morphologic differentiation with the expression of microvilli (M) and tight junctions (TJ). The cells were polarised with a typical asymmetric morphology, and glycogen deposits and brush border microvilli were present. Integrity of the monolayer was confirmed by the presence of tight junctions in the apical zone, and interdigitation (I) as well as desmosomes along the cells.

Transport of Iron Cell Monolayers

Iron transport experiments were carried out when the Caco-2 cell and INT 407 monolayers reached the

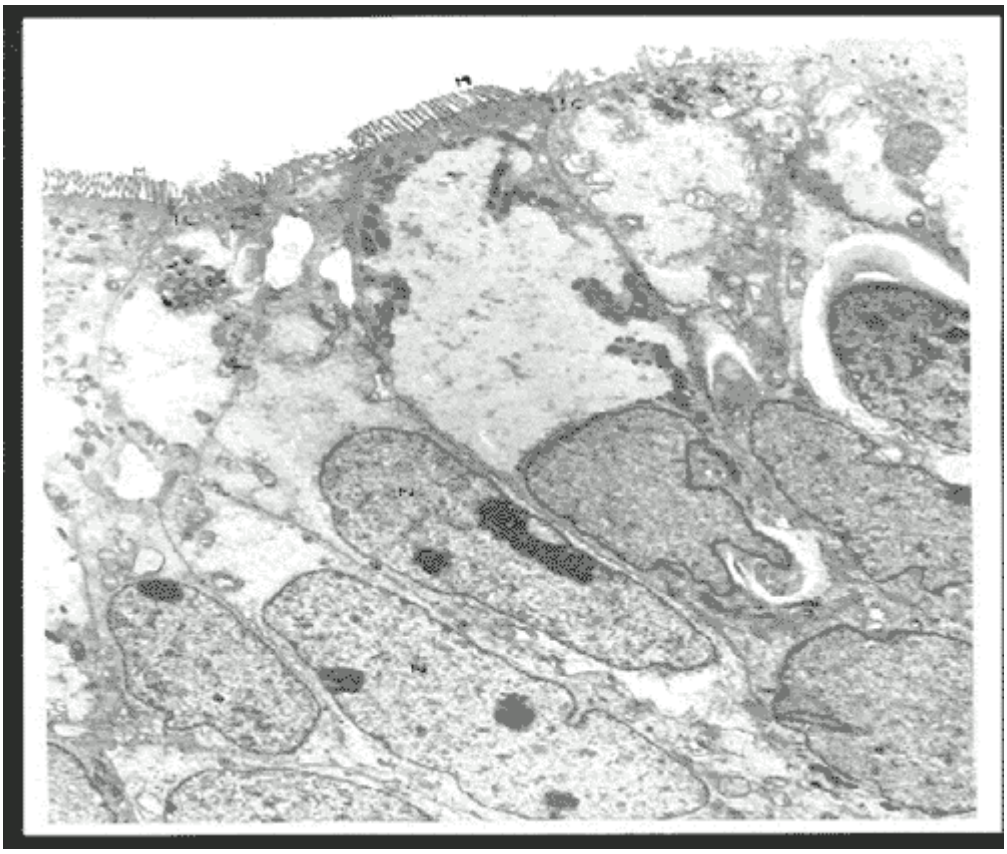

Figure 4: A TEM of Caco-2 cells: (X 89000)
M: microvilli JC: Junctional Complex

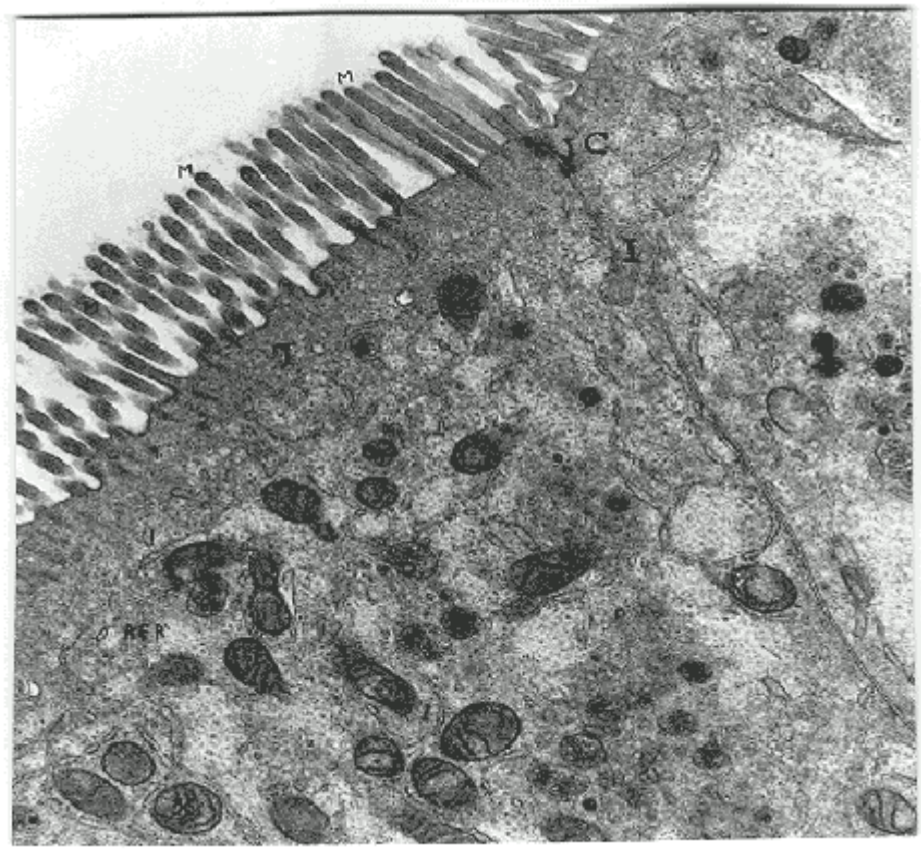

Figure 5: A TEM of Caco-2 cell showing enterocytic-like differentiation features. M:microvilli, T:terminal web, JC:Junctional complex, I:interdigitation

highest percent of phenol red diffusion,< 2.5% per hour , together with maximal TEER (250 Ω cm^2) for Caco-2 cells. Results in Fig.6 and 7 shows that ^{59}Fe could be transported across both Caco-2 and INT 407 monolayers from three different sources at varying degrees. A much higher percentage of iron was transported across INT 407 monolayer from the upper to the lower chambers as compared to Caco-2. However similar pattern of iron transportation from the three different sources were observed in that more ^{59}Fe-lactoferrin and ^{59}Fe-citrate were transported than ^{59}Fe-transferrin. In all cases the amount of iron transported across Caco-2 monolayers formed only a small proportion (< 3%) of the total iron added, a finding that does, however, mirrors the limited absorption of dietary iron that occurs *in vivo.* Unlike this finding, iron was transported as much as 40 % of added iron across the INT 407 monolayers non-selectively. Further

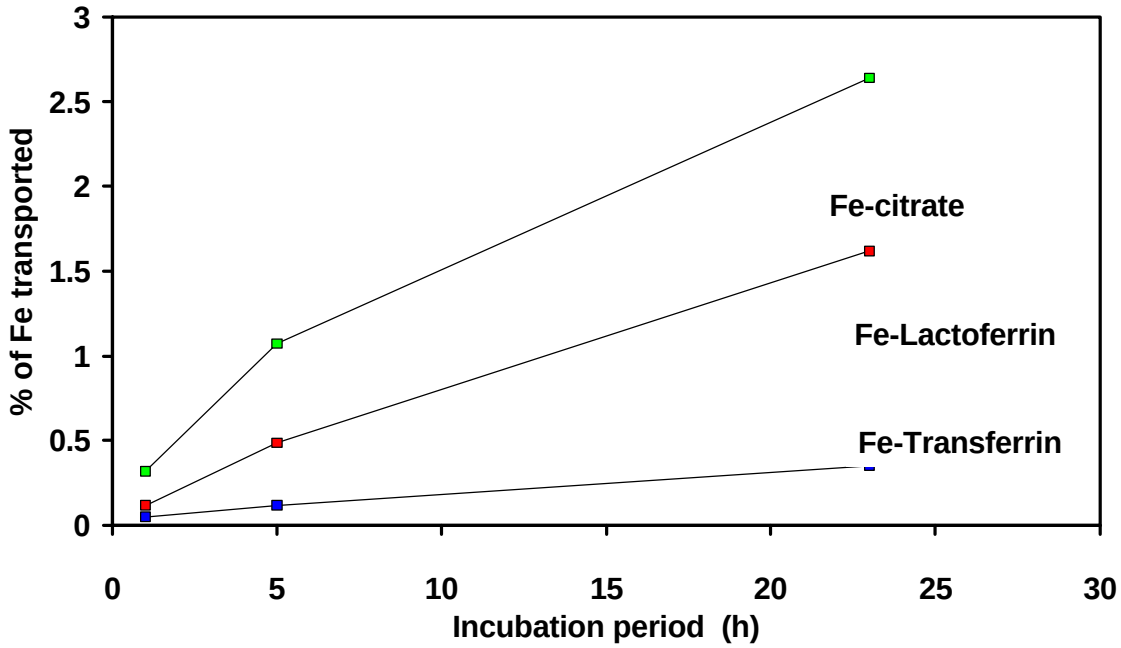

Figure 6: Transport of iron across the Caco-2 cell monolayers grown in the Transwell bicameral chambers

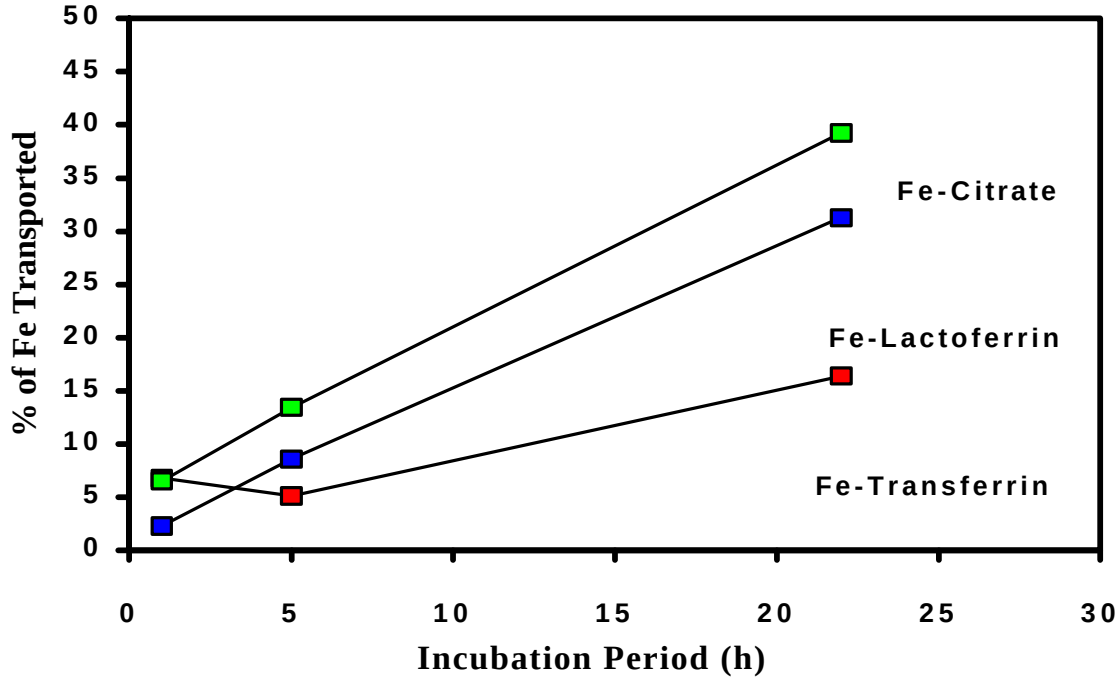

Figure 7: Transport of iron across INT 407 cell monolayers cultured in Transwell bicameral chambers

Table 1: Transport of lactoferrin and Transferrinacross Caco-2 and INT 407 cell monolayers cultured in bicameral chambers

Incubation Time (h)	% of radioactivity detected in the lower chamber			
	^{125}I-Lf		^{125}I-Tf	
	Caco-2	INT 407	Caco-2	INT 407
1	0.3±0.03	6.3+0.12	0.4+0.03	4.8+0.12
5	1.1± 0.08	12.2± 0.83	1.4± 0.09	10.3± 0.98
23	3.5± 0.10	42.1± 0.5	4.5± 0.11	35.4± 3.4

work on the iron accumulated within the Caco-2 cells have shown that only less than 5% of total added iron was retained and thus making the total iron uptake (transported and retained) greatest when iron bound to lactoferrin followed by citrate and much less by transferrin.

Transport of Iron-Binding Proteins

When the passage of lactoferrin or transferrin proteins across Caco-2 monolayers was studied, it was found that 3.5% and 4.5% of the ^{125}I label originally associated with lactoferrin and transferrin respectively had traversed the monolayer in 23 h (Table 1). However, for both proteins only about 20% of the radioactivity that had traversed the monolayer was TCA-precipitable and as shown by SDS-PAGE, indicating that most of the protein was degraded when crossing the cells. Unlike Caco-2 cells, as much as 42% and 35% of ^{125} I-lactoferrin and ^{125}I-transferrin were transported across INT 407 monolayers at the end of 23 h incubation period, and almost all of these proteins were TCA-precipitable.

DISCUSSION

The human colon carcinoma cell line, Caco-2 grown on collagen coated polycarbonate membrane mounted in Transwell bicameral chambers was found to exhibit structural and functional differentiation features normally associated with the characteristics of mature enterocytes as have been reported by earlier workers (Hidalgo *et al.*,1989; Alvarez-Hernandez *et al.*, 1991). The Caco-2 cell monolayers were seen from transmission electron micrograph to be covered by brush border micrivilli. The structural and functional differentiation of the brush border microvilli is associated with polarisation of the epithelial monolayers as displayed by the presence of tight junctions and the appearance of desmosomes which are known to be specific features of polarised epithelia (Cereijido, 1978).

The electron micrographs of these cells were taken on day 17 after seeding on to the polaycarbonate membranes, when they have already become columnar in shape. Therefore changes in cell dimensions and formation of these features

during earlier stages of culture were not examined. However, Hidalgo *et al.* (1989) had reported that the height of the cell had increased as much as 489% from day 3 to day 16. The microvilli started to appear on day 3 but were poorly organised.

The development of polarity of the Caco-2 monolayers was determined from measurements of TEER across the monolayers. The TEER values of day 1 to post confluence obtained in this study were similar to those observed by Alvarez Hernandez (1991) but slightly higher than the values obtained by Hidalgo (1989) at day 8 which then remained almost constant up to day 18. The TEER values increased above 250 Ωcm^2 on day 16 suggesting that Caco-2 monolayers had then formed a tight barrier. The tightness of the barrier was further confirmed by the impermeability of the cells to phenol red. The minimum percentage of phenol red diffusion obtained was 5.8 ± 0.4 % from day 14.. Halleux and Schneider (1991) obtained the minimum percentage of phenol red diffusion as 6 % after day 7 of cultivation, suggesting that confluency was reached a few days earlier. The use of different passages of cells plus a different type of membrane could contribute to the slight difference.

Unlike Caco-2 cells, INT 407 cells did not differentiate in culture after cultivation of more than 20 days. Results from phenol red exclusion indicate that these cells formed a complete monolayer, in fact the minimum percentage of phenol red diffusion (6.9 ± 0.2%) was reached after only 7 days in culture. However TEER values across these cells did not increase and never reached more than 90.5 ± o.3 Ωcm^2. Therefore, although on the one hand INT 407 cells could be cultured on the polycarbonate membrane and could form a monolayer, these cells nevertheless could not differentiate as Caco-2. The finding is not totally unexpected because attempts to either culture intestinal epithelial cells or establish cell lines derived from enterocytes have not been successful (Qudroni *et al.* 1979; Raul *et al.* 1978). Similar reults were obtained by Chantret *et al.* (1988) when using various colon carcinoma cell lines and most of these cells do not develop enterocytic differentiation in culture, with the exception of a few cell lines, inmcluding Caco-2 cells.

Having established that Caco-2 cultured in the Transwell inserts had undergone enterocytic differentiation, they were then used as a model of enterocytes to study the transport of iron and proteins (lactoferrin and transferrin) across the brush border into the cells as well as across them. Undifferentiated INT 407 cells were used as the control, and since INT 407 cells also formed good monolayers, the diffrence in the pattern of transport could be attributed to special features of diffrentiated Caco-2 cells.

Transport of lactoferrin and transferrin across Caco-2 monolayers to the lower chamber is similar, with only 4% of the proteins being transported within 24 h. Furthermore most of these proteins was TCA soluble, indicating that these proteins had been degraded and only < 20 % was still intact. With the present set up, it is not known exactly where degradation has

taken place, either in the cytosol or as the proteins cross over the basal membrane. In contrast almost half of the proteins were found to have been transported transcellularly across INT 407 cells. At the end of 23 h, as much as 42 % of lactoferrin and 49 % of transferrin was detected in the lower chamber, and unlike the earlier finding with Caco-2 cells, only 43 % and 37 % of these proteins were degraded. These findings suggest that neither lactoferrin nor transferrin was transported across Caco-2 cell monolayers and the passage of these proteins across INT 407 cells could occur through intercellular spaces rather than transcellularly.

Transport of lactoferrin bound iron by Caco-2 was comparable to transport of iron presented as ferric citrate, and greater than that of iron transported from transferrin. If iron transport across the cells, rather than release from lactoferrin is the rate-limiting step, then Fe-lactoferrin and ferric citrate would be expected to behave similarly. In contrast, since Caco-2 cells express transferrin receptors (Schreider & Halleux, 1991), iron bound to transferrin may be taken up by endocytosis of the transferrin- iron complex and used for cell metabolism. The fact that rather more iron from transferrin than from lactoferrin was found within the cells, despite the minimal transport of transferrin-iron across the monolayer, tends to support this hypothesis, though proof would require examination of the intracellular distribution of iron. As there was little evidence that neither lactoferrin nor themselves are transported across the cell monolayers, this suggests that iron from lactoferrin detected across the monolayer was due to the release of this iron from membrane bound lactoferrin and not due to it being transported by the protein itself. If this is true, then iron from lactoferrin or citrate would be expected to behave similarly and being taken up by the cell following the same route.

These findings are in line with the *in vivo* study of iron absoption from ^{58}Fe-labelled bovine lactoferrin in newly born infants which did not demonstrate any difference from the absoption of ferrous chloride (Fairweather-Tait *et al.,* 1987). These workers concluded that lactoferrin-bound iron was handled by the body in exactly the same way as other non-haem dietary iron. In addition other *in vivo* studies show that lactoferrin neither inhibits nor enhances absorption of irorganic iron (Fransson *et al.*, 1983: Davidson *et al.,* 1990).

Differentiated Caco-2 cells thus provide an *in vitro* model of the intestinal barrier for the transport study of various substances (Hidalgo *et al.* 1989: Halleux & Schneider, 1991). Therefore, Caco-2 cells can be a useful *in vitro* model for studying bioavailability of iron such as dietary factors regulating its absorption and indeed have been used for this purpose by others (Alvarez-Hernandez *et al.* 1991, Alvarez-Hernandez *et al.* 1994, Glahn *et al.* 1996).

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