Biochimica et Biophysica Acta, 1070 (1991) 205–208

BBAMEM 75441

Caco-2 cell line: a system for studying intestinal iron transport across epithelial cell monolayers

Xavier Alvarez-Hernandez, Gary Michael Nichols, and Jonathan Glass

Department of Medicine, Louisiana State University Medical Center, Shreveport, LA (U.S.A.)

(Received 6 May 1991)

Key words: Intestine; Iron transport; Caco-2

Iron transport across polarized intestinal epithelium was studied by using Caco-2 cells grown in bicameral chambers. When cells were grown under conditions of low, normal, or high iron concentration not only was the iron content of the cells markedly altered but the low iron cells exhibited a nearly 2-fold increase in transepithelial electrical resistance (TEER). ^{59}Fe uptake from the apical surface into cells and transport into the basal chamber was affected both by the valency of the iron and the iron status of the cells. Uptake from ^{59}Fe(II)-ascorbate was about 600 pmol ^{59}Fe/h per mg protein, increased about 2-fold in low iron cells, and was about 13–200-fold greater than uptakes from ^{59}Fe(III) chelated to nitrilotriacetic acid, BSA, or citrate. Transport into the basal chamber from ^{59}Fe(II)-ascorbate was 3.7 ± 1.7 pmol/h per cm^2 for Fe-deficient cells vs. 0.72 ± 0.1 pmol/h per cm^2 for normal-Fe cells and from ^{59}Fe(III)-BSA 1.1 ± 0.2 pmol/h per cm^2 vs. 0.3 ± 0.03 pmol/h per cm^2 for deficient vs. normal iron cells, respectively. The greater transport of iron both from Fe(II) and in iron deficient cells supports the use of the Caco-2 cells as a model for iron transport.

Introduction

Total body iron balance is regulated by iron absorption which occurs primarily in the proximal small intestine. The regulation is unique in nutrient uptake in that there are no regulated excretory pathways for iron and the intestinal cell is sensitive both to total body iron stores and levels of erythropoiesis [1,2]. The systems which have been used previously to study mechanisms of iron absorption are either composed of multiple cell types as with isolated gut loops [3–7] or have examined uptake into vesicles isolated from the apical (lumenal) surface of small intestine mucosa [8–10]. While there are studies involving primary cell cultures of cells derived from intestinal mucosa, these preparations, which do not form tight cell monolayers and may be composed of more than one cell type, do not permit analysis of transport of iron from the apical to the basal-lateral surface. The Caco-2 cell line has been used to examine a variety of intestinal functions [11] and is attractive to use for the study of iron transport for several reasons: the cells form a highly polarized monolayer, exhibit many of the features of small intestine cells, and have been used to demonstrate vectorial transport [11]. In the present study we examine the utility of Caco-2 cells grown on porous membranes in bicameral chambers to study transport of iron from the apical to the basolateral surface and demonstrate the transport to be responsive both to the iron status of the cells and to the valency of iron.

Material and Methods

Caco-2 cells, from American Type Culture Collection No. HTB37 (Rockville, MD), were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum (FBS, (Gibco, Gaithersburg, MD)), 1% non-essential amino acids, and antibiotics/antimycotic (100 u/ml Penicillin-G, 100 u/ml Streptomycin and 250 ng/ml Fungizone). To culture cells under low iron conditions Dulbecco's MEM without added iron (Gibco) was used supplemented with iron depleted FBS prepared by titrating the FBS to pH 4.5 in the presence of 300 g/l Chelex (Sigma, St. Louis, MO); after 2 h the pH was increased to pH 7.4 with

This work was supported in part by grant DK-41279 from the National Institutes of Health.

Correspondence: X. Alvarez-Hernandez, Section of Hematology/Oncology, Louisiana State University Medical Center, 1501 Kings Highway, Shreveport, LA 71130, U.S.A.

NaOH and the FBS allowed to sit overnight in the presence of Chelex. To culture cells under high iron conditions, the medium was made 65 μM in Fe(III) by the addition of Fe(III)-NTA. Cells were grown in Transwell bicameral chambers with 3 μm pore size membrane (Costar, Cambridge, MA) coated with collagen. The collagen film was applied to the filter as 50 μl of collagen solution (3 mg/ml, 60% ethanol; rat tail, type I, Collaborative Research Products) and then the Transwells were inverted and dried under sterile laminar air flow. Formation of a monolayer was monitored by measuring the transepithelial electrical resistance (TEER) with a Millicell electrical resistance system (Millipore) [11]. Iron uptake was measured only after TEER had risen to a level indicating the formation of an intact monolayer [11–13]. ^{59}Fe ($^{59}FeCl_3$, 5–85 Ci/g, NEN-DuPont) was added as Fe(II)-ascorbate at an Fe/ascorbate [14] ratio of 1 : 1000 or as Fe(III) chelated to: NTA (1 : 4) [15,16], citrate (1 : 3000) [6,17], BSA [18], or transferrin [19]. The ^{59}Fe chelates were added at 500 nM Fe to the apical or upper chamber in 50 mM Hepes-buffered saline solution (HBS) (pH 7.4), prepared from 193 mM Hepes diluted with an isotonic salt solution consisting of 130 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgSO_4$. The buffer in the basal or lower chamber was HBS made 50 μM in human apo-transferrin. Uptake was allowed to proceed for 60 min, the basal chamber buffer harvested, the cells washed three-times with HBS and removed from the polycarbonate membranes with 0.5% trypsin. Radioactivity in the cells, apical and basal chambers was determined in a Compugamma LKB gamma counter. Iron concentrations of media, FBS and cells were estimated by atomic absorption spectroscopy (AAS) in a Perkin-Elmer AAS equiped with an argon furnace. The iron concentration of the iron depleted FBS and medium was always less than 0.1 μM. Caco-2 cell ferritin was measured with a Elisa Remco-kit for human ferritin that uses as a standard liver ferritin. Electron microscopy was performed as described elsewhere [10].

Results

Formation of Caco-2 cell monolayers

Similarly to previous reports [10,12] Caco-2 cells grown on polycarbonate membranes demonstrated a gradual increase in TEER with maximal TEER being reached at about day 18 of culture (Fig. 1). Electron microscopic analysis of Caco-2 cells after day 18 showed cell monolayers with a highly polarized structure, abundant microvilli in the apical surface, well developed tight junctions, glucagon storage grains, and clathrin-coated pits (micrographs not shown). At the time of maximal TEER the monolayer was sealed to passage of dextran blue (molecular mass $2 \cdot 10^6$ Da), ^{125}I-labeled BSA (molecular mass 66 000 Da), and phenolsulphonphthalein (molecular mass 354 Da) with leakage of these probes of less than 0.25% per h in either direction (data not shown).

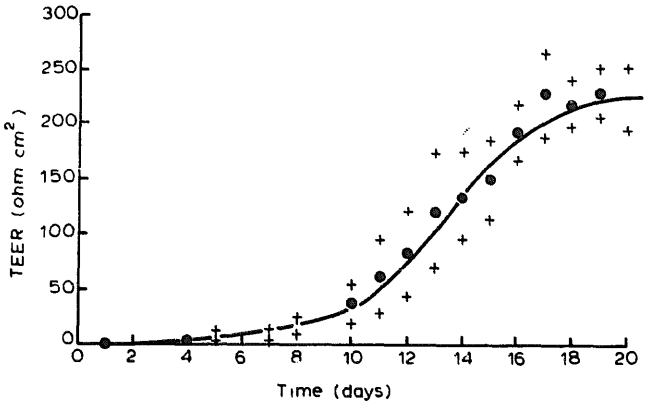

Fig. 1. Transepithelial electrical resistance of Caco-2 cell monolayers. Caco-2 cells were grown in transwells bicameral chambers as described in the methods in normal iron containing media for up to 20 days. Transepithelial electrical resistance (TEER) was measured at the indicated times. The TEER expressed as ohm cm^2 are means (●) ± S.D. (+) of 10–12 inserts per day.

Culturing cells in iron-depleted or iron-enriched medium significantly altered several parameters as detailed in Table I. The Fe content of the cells was markedly affected with cells cultured in low Fe conditions containing about half the iron as cells grown under normal iron conditions while cells grown under high iron conditions had a 4-fold increase in iron content. Ferritin content was also affected: the iron-deficient cells had no detectable ferritin by the ELISA assay while the iron-loaded cells had a several hundred fold increase in ferritin over normal and showed a positive reaction for hemosiderin when stained with

TABLE I

The effect of iron status of Caco-2 cells on iron and ferritin content and TEER

Caco-2 cells were cultured either under low iron ([Fe] < 0.1 μM), normal iron ([Fe] = 1.1 μM), or high iron ([Fe] = 65 μM) conditions. At day 18 iron content, ferritin, and TEER of the cells were measured as described in Methods. The iron and ferritin contents were measured in cells grown on 3 cm diameter plastic dishes to obtain sufficient cells required for these measurements. Shown are the means ± S.D. for six plates for iron and ferritin content, and 44, 52, and 12 inserts for TEER for low, normal, and high Fe, respectively. n.d., not detected.

	Low iron	Normal iron	High iron
Iron content (pmol Fe/mg protein)	11.0 ± 3.5	25 ± 7	114 ± 23
Ferritin (pmol ferritin/mg protein)	n.d.	0.53 ± 0.16	269 ± 47
TEER (ohm cm^2)	497 ± 66	246 ± 25	190 ± 25

TABLE II

^{59}Fe uptake into Caco-2 cells as a function of iron status of the cells

The uptake of ^{59}Fe from the various sources was determined in Caco-2 cell monolayers grown on 3 cm^2 plastic dishes and is expressed as picomoles of Fe/mg protein per h. Values are the mean ± S.D. of at least six replicates of three independent experiments.

^{59}Fe offered at 500 μM as:	Cell monolayer status		
	Fe deficient	Fe normal	Fe high
Fe^{+2}-ascorbate	1213 ± 156	614 ± 49	-
Fe^{+3}-NTA	79 ± 9	45 ± 4	34 ± 3
Fe^{+3}-BSA	35 ± 4	27 ± 2	-
Fe^{+3}-citrate	4 ± 1	3 ± 1	1 ± 1
Fe^{+3}-transferrin	5 ± 1	3 ± 1	2 ± 1

Prussian blue. The TEER was also affected with the iron-deficient cells exhibiting a 2-fold increase in TEER at day 11 with no abnormalilties of TEER occurring in the iron-loaded cells.

^{59}Fe uptake and transepithelial transport

^{59}Fe was offered to the apical surface of the Caco-2 cells either as Fe(II)-ascorbate or Fe(III) chelated either to NTA, BSA, citrate or transferrin. As seen in Table II while uptake into the cells occurred both from Fe(II) and Fe(III) sources, nearly 15-fold more ^{59}Fe was taken up from Fe(II)-ascorbate than any of the Fe(III) donors. ^{59}Fe uptake into the cells was approximately doubled by growth under iron-deficient conditions. Uptake into the iron loaded cells was not significantly affected.

Fig. 2 shows ^{59}Fe transport from the apical to the lower chamber for normal and iron deficient monolayers with either ^{59}Fe-ascorbate or ^{59}Fe-BSA in the apical chamber. After a lag of about 15 min there appeared to be linear transport of ^{59}Fe into the basal chamber. As with cell uptake, transport of ^{59}Fe offered as ^{59}Fe(II)-ascorbate was greater than ^{59}Fe(III)-BSA with rates of 0.72 ± 0.1 pmol/h per cm^2 versus 0.3 ± 0.03 pmol/h per cm^2 observed for ^{59}Fe-ascorbate and ^{59}Fe-BSA respectively in cells with normal iron content. Transport across the iron deficient cells was increased for both iron sources with rates of 3.7 ± 1.7 pmol/h per cm^2 observed for ^{59}Fe-ascorbate and 1.1 ± 0.2 pmol/h per cm^2 for ^{59}Fe-BSA.

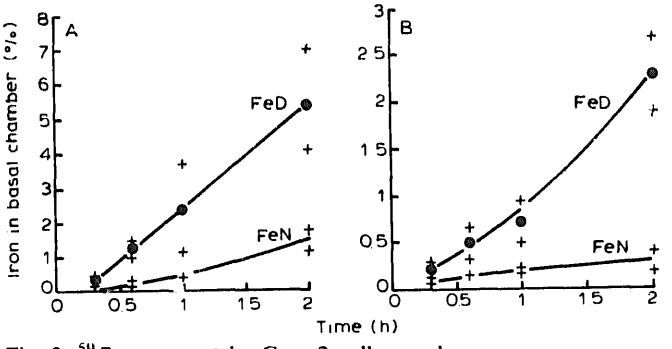

Fig. 2. ^{59}Fe transport by Caco-2 cell monolayers grown on porous membranes in bicameral chambers. Caco-2 cells were grown under normal iron (FeN) or iron deficient (FeD) conditions until maximal TEER was reached. ^{59}Fe at 500 μM was then offered on the apical side either as ^{59}Fe(II)-ascorbate (A) or as ^{59}Fe(III)-BSA (B). Aliquotes were removed from the basal chamber at the indicated times, replaced with fresh buffer and ^{59}Fe radioactivity determined. Presented is the percentage of total radioactivity transported to the basal chamber (mean (●) ± S.D. (+) of two experiments with each point in triplicate).

Discussion

A major handicap in the study of iron absorption has been the lack of an *in vitro* cell culture model of homogenous enterocytes that could form a polarized barrier. The system based on Caco-2 cells eliminates many of variables that could obscure the cellular and molecular mechanisms operating in metal absorption. The Caco-2 cell monolayers, grown in our laboratory in bicameral chambers on porous membranes, are morphologically highly polarized as seen by electron microscopy. The cells present abundant microvilli and well-defined tight junctions with all features of the zona adherence present. Caco-2 monolayers form a tight barrier as shown by a TEER that increases above 250 ohm cm^2. The tightness of the barrier was confirmed by the inpermeability of the cells to phenosulphonphthalein. The formation of a tight barrier makes the Caco-2 cells an excellent model for the study of transcellular transport.

In the present study we have demonstrated that the Caco-2 cells take up and transport ^{59}Fe and that both processes are more rapid with Fe(II) than Fe(III). The observed uptake rates in Table II when expressed as pmol/mg protein per min are similar to the rates seen for uptake into apically derived vesicles [3] therefore suggesting that similar processes are being observed. Although *in vivo* uptake from Fe(III) may occur [6,9,10,18] our finding is in accord with studies that suggest Fe(II) is a more optimal source of iron. That the differences in transport into the lower chamber were not as great as the uptake into the cells suggests that in this model the transfer phase and not the uptake phase may be rate-limiting. To exclude restriction of iron binding sites in the basal chamber as rate limiting in the transfer phase, the physiological iron aceptor apo-transferrin was supplied at 50 μM which allowed for a 200–1000-fold molar excess of binding sites to ^{59}Fe transported.

It is also apparent from these studies that not only are the Caco-2 cells susceptible to changes in iron status by culturing the cells with different iron concentrations in the media, but that these changes affect cell function. Most pertinent to the model is that both iron uptake into the cell and transport across the cell is

greater in the iron-deficient cells and decreased in the iron-loaded cells. The preference for Fe(II) and the effects of iron deficiency and iron loading are in agreement with observations in humans and animals and serves to verify the Caco-2 model as appropriate for the study of iron uptake. Although it is possible that other polarized cell lines could exibit iron uptake from non-transferrin bound iron, the Caco-2 cell system is presented as an appropiate model for intestinal transport because the cells are derived from the intestine and have morphological and enzymatic properties of intestinal epithelium.

Intriguing is the observation that the iron-deficient cells exhibited a higher TEER. Preliminary electron microscopy studies demonstrate in the iron-deficient cells larger tight junctions with an apparent increase in the number of strands forming the tight junctions. While it is difficult to understand how these structural changes translate into increased Fe uptake and transport, the changes give further support that the observed iron transport into the lower chamber is by a transcellular rather than paracellular mechanism.

References

1 Conrad, M.E. (1987) in Physiology of the Gastrointestinal Tract (Johnson, L.R., ed.), pp. 1437–1453.Raven Press, New York.

2 Flanagan, P.R. (1990) in Iron Transport and Storage (Ponka, P., Schulman, H.M. and Woodworth, R.C., eds.), pp. 247–261.

3 Simpson, R.J., Osterloh, K.R.S., Raja, K.B., Snape, S.D. and Peters, T.J. (1986) Biochim. Biophys. Acta 884, 166–171.

4 Levey, J.A., Barrand, M.A., Callingham, B.A. and Hider, R.C. (1988) Biochem. Pharmacol. 37, 2051–2057.

5 Conrad, M.E., Parmley, R.T. and Osterloh, K. (1987) J. Lab. Clin. Med. 110, 418–426.

6 Marx, J.J. and Aisen, P. (1981) Biochim. Biophys. Acta 649, 297–304.

7 Savin, M.A. and Cook, J.D. (1980) Blood 56, 1029–1035.

8 Simpson, R.J. and Peters, T.J. (1986) Biochim. Biophys. Acta 856, 115–122.

9 Teichmann, R. and Stremmel, W. (1990) J. Clin. Invest. 86, 2145–2153.

10 Simpson, R.J. and Peters, T.J. (1984) Biochim. Biophys. Acta 772, 220–226.

11 Audus, K.L., Bartel, R.L., Hidalgo, I. and Borchardt, R.T. (1990) Pharmac. Res. 7, 435–451.

12 Hidalgo, I.J., Raub, T.J. and Bourchardt, R.T. (1989) Gastroenterology 96, 736–749.

13 Pinto, M., Robine-Leon, S., Appay, M.D., Kelinder, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) Biol. Cell 47, 323–330.

14 Plug, C.M., Dekker, D. and Bult, A. (1984) Pharmaceutish Weekblad Sc. Ed. 245–248.

15 Martell, A.E. and Smith, R.M. (eds.) (1974) in Critical Stability Constants, Vol. 1, 139–143, Plenum Press, New York.

16 Kontoghiorghes, G.J. (1990) Arzneim.-Forsch. Drug Res.40, 1332–1335.

17 Spiro, T.G., Bates, G.W. and Saltman P. (1967) J. Am. Chem. Soc. 89, 5559–5562.

18 Wollenberg, P. and Rummel, W. (1987) Naunyn-Schmiedeberg's Arch. Pharmacol. 336, 578–582.

19 Foley, A.A. and Bates, G.W. (1988) Biochim. Biophys. Acta 965, 154–162.