A Lipophilic Iron Chelator Can Replace Transferrin as a Stimulator of Cell Proliferation and Differentiation

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ABSTRACT Of the different growth supplements used in chemically defined media, only transferrin is required for differentiation of tubules in the embryonic mouse metanephros. Since transferrin is an iron-carrying protein, we asked whether iron is crucial for tubulogenesis. Differentiation of metanephric tubules both in whole embryonic kidneys and in a transfilter system was studied. The tissues were grown in chemically defined media containing transferrin, apotransferrin, the metal-chelator complex ferric pyridoxal isonicotinoyl hydrazone (FePIH), and excesses of ferric ion. Although we found that apotransferrin was not as effective as iron-loaded transferrin in promoting proliferation in the differentiating kidneys, excess ferric ion at up to 100 μM, five times the normal serum concentration, could not promote differentiation or proliferation. However, iron coupled to the nonphysiological, lipophilic iron chelator, pyridoxal isonicotinoyl hydrazone, to form FePIH, could sustain levels of cell proliferation and tubulogenesis similar to those attained by transferrin. Thus, the role of transferrin in cell proliferation during tubulogenesis is solely to provide iron. Since FePIH apparently bypasses the receptor-mediated route of iron intake, the use of FePIH as a tool for investigating cell proliferation and its regulation is suggested.

Transferrin is an iron carrier required for proliferation of many cell types in chemically defined media (3, 43, 48). It has also been shown to stimulate embryonic development (11, 12, 23). For its actions, transferrin must first bind to its receptor. The transferrin-receptor complex is then rapidly internalized via coated pits, transferrin looses its iron in acidic endosomes, and finally apotransferrin is returned to the extracellular space (8, 25–27, 42, 39, 47). Morphological evidence for this pathway is now available (5, 10, 13, 18, 20). The growth-stimulating effects of transferrin apparently require the presence of its receptor. Interference with the metabolism of transferrin with antibodies to the receptor can affect the cell cycle and growth (45). Furthermore, the expression of transferrin receptor at the cell surface is related to cell proliferation (10, 17, 22, 40, 41, 44). Thus, it could be proposed that the transferrin-receptor interaction has an independent direct effect on proliferation not related to iron transport, although some previous studies based on either replacement of transferrin with iron salts (19, 36) or removal of iron from transferrin (31) suggest that the iron is essential in promoting proliferation. However, these studies may be plagued with the difficulties of decreased affinity for apotransferrin by the receptor (8, 21) or by uncertainties in the removal of absolutely all iron or transferrin from the media. Further-

more, iron can be toxic and is not readily internalized without transferrin (2), which explains why iron salts fail to stimulate proliferation of many cell types (2, 43, 45, 48). Recently, Ponka and co-workers (32, 33) described a lipophilic iron chelator that can be used for iron mobilization. This chelator, pyridoxal isonicotinoyl hydrazone (PIH),[1] was found to bring out iron with the bile from overloaded liver in hypertransfused rats (7). The iron chelate complex of PIH was later shown to deliver radio-iron intracellularly to reticulocyte heme (34). Therefore, in spite of reports that metal chelators, including PIH, cannot stimulate iron intake in other cells (14, 29, 30, 45), it was thought that the properties of ferric pyridoxal isonicotinoyl hydrazone (FePIH) may be used to investigate the role of iron in cell proliferation.

In the present study, we demonstrate that FePIH can replace transferrin in a chemically defined medium as a stimulator of proliferation of differentiating embryonic cells. Moreover, the response is physiological since the cells in the model system used convert from undifferentiated mesenchymal cells

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[1] *Abbreviations used in this paper:* FePIH, ferric PIH; IMEM, improved Eagle's minimum essential medium; PIH, pyridoxal isonicotinoyl hydrazone.

to epithelial cells and form three-dimensional branched kidney tubules. This organ culture model is especially suited for analyses of transferrin metabolism since other growth factors are not required or used in the system. Our preliminary studies on the effect of FePIH have been reported in an abstract (26).

MATERIALS AND METHODS

Reagents: Isonicotinoyl hydrazide, pyridoxal, substantially iron-free human transferrin, picolinic acid, and nitrilotriacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium perchlorate was purchased from Koch-Light Laboratories (Colnbrook, UK), and ferric chloride hexahydrate was purchased from E. Merck AG (Darmstadt, Federal Republic of Germany). A 0.2 mM solution of (methyl-H3) thymidine with an activity of 25 Ci/mmol was obtained from Amersham International (Buckinghamshire, UK). All other reagents were reagent grade, and the water used was doubly glass-distilled. All commercially available reagents were used without further purification.

PIH was synthesized as described by Ponka and co-workers (32, 33). In a boiling water-bath, PIH precipitates within 5 min upon mixing equimolar quantities of nearly saturated solutions of isonicotinoyl hydrazide and pyridoxal in 0.1 M sodium acetate buffer, pH 4.5. An 80% yield remained after filtration and extensive washing with cold water. PIH can be solubilized in dilute acid.

FePIH was prepared by mixing ferric citrate with PIH. Ferric chloride was dissolved in a 10-fold excess of citric acid. This solution was mixed with a twofold molar excess of PIH in citric acid. The brown FePIH precipitated when the solution was neutralized. The precipitate was collected by centrifugation and washed five times with water.

In experiments designed to compare transferrin with apotransferrin, highly pure human transferrin kindly donated by Dr. P. Aisen (Albert Einstein College of Medicine, New York) and apotransferrin prepared by us from that transferrin were used. The transferrin was carefully rendered iron-free by dialysis against 0.1 M citrate acetate at pH 4.5 in the cold as described by Aisen et al. (1). The solution was dialyzed extensively against water. Iron was added back to apotransferrin with ferric citrate in the presence of carbonate at pH 8.6. The iron-saturated preparations showed maximal absorption at 470 nm and had a salmon-pink color. The color disappeared upon removal of iron. The degree of iron saturation of the apotransferrin was estimated by absorption data to be <4% (1, 20). Although we carefully tried to control the iron of the medium, we cannot exclude that the iron-saturation of the transferrins changed during the organ cultures. The iron saturation was not measured after culture.

Organ Culture: All tissues were aseptically and microsurgically isolated from 11- to 12-d-old CBA T₆T₆ × C57 black hybrid mouse embryos of mothers sacrificed by cervical dislocation. The age of the embryos was counted from the day of vaginal plug, day 0.

Two types of tissue cultures were performed. First, transfilter experiments were carried out as previously described (15, 16, 37). One or three metanephric mesenchymes from 11-d-old embryos were placed on a Nuclepore filter (Pleasanton, CA) with a pore size of 1.0 μm. On the lower surface of the filter a heterotypic inducer for tubulogenesis, spinal cord, was attached with agar. The tissues were usually cultured on stainless steel, Trowell-type screens for 2 or 3 d (9, 38). However, in experiments where the iron content of the media was a variable, the tissues were cultured in plastic Auerbach dishes instead of stainless steel grids. In these dishes a plastic bridge was placed across a well filled with medium. Holes in the bridge allowed access of the medium to the tissues. The filters were placed across the holes so that the tissues were situated at the medium surface. In the second type of cultures, whole kidneys from 11- to 12-d embryos were cultured from 1 to 3 d on Trowell-type screens or Auerbach bridges.

All culture media were chemically defined. The negative control medium was improved Eagle's minimum essential medium (IMEM) (35). This medium contained 2 μM ferric chloride. The positive control medium was IMEM supplemented with human transferrin (Sigma Chemical Co.) at 50 μg/ml (11). Penicillin and streptomycin were used throughout, and 4 mM glutamine was added when the experiments were initiated.

Experimental media were made by adding aliquots of an aqueous reagent solution to concentrated stock IMEM before proper dilution at the time of the experiment. Ferric chloride, picolinic acid, and nitrilotriacetic acid easily form aqueous stock solutions of adequate concentration. FePIH, however, is more difficult to solubilize. A 1-mM solution could be made after dilute acidification. The neutralized solution was heated slightly at the time of the experiment in order to completely dissolve any precipitate. No precipitate was ever noticed in the starting culture media. The FePIH concentration was never >40 μM in any experiment.

In the transferrin versus apotransferrin experiments, only the pure transferrin was used. The effect of iron in the media was also studied. Two methods of removing contaminating iron were attempted. After reduction of ferric ion,

chemical removal of all divalent cations followed by readdition of all but ferric chloride yielded a toxic medium. Attempts to chelate the iron in IMEM with nitriloacetic acid and picolinic acid were also made. Low concentrations of these chelators had no effect (data not shown), while higher concentrations of these acids changed the pH or ionic strength of the media and were not used. We report the effect observed when ferric chloride was simply deleted from the media.

Thymidine Incorporation: The rate of DNA synthesis was measured by radioactive thymidine incoporation into the trichloroacetic acid-precipitable material, and radioactive counts were normalized for DNA content (28). In transfilter experiments, the spinal cord fragment was removed by gentle scraping before the subculture in radioactive media. Tritiated thymidine was added to the media to a final concentration of 4 μM and a final radioactivity of 20 μCi/ml, and the cultures were continued for 3 h. The tissues were then placed in 500 μl of water and stored at −20° until assays for DNA content and radioactivity could be carried out.

Histology: For light microscopy, the whole kidneys and transfilter explants were fixed in Zenker's solution. Paraffin sections of these tissues were stained with hematoxylin-eosin. Whole mounts of single mesenchyme transfilter experiments were fixed in 10% formalin after removal of the spinal cord. The whole mounts were also stained with hematoxylin-eosin. The number of tubules in these whole mounts was then counted in order to quantify the degree of tubulogenesis that had occurred (11, 38).

RESULTS

Effect of Ferric Ion in the Absence of Transferrin

We first examined the role of iron in proliferation and differentiation by increasing the medium concentration of ferric chloride in the absence of transferrin. On the first day, the proliferation rate of whole kidneys from 11½-d-old embryos was high regardless of the composition of the media (Fig. 1 *A*). On days 2 and 3, there was a significant difference between the rate of proliferation of the transferrin supplemented medium and that of all other media. While transferrin-supplemented medium continued to support a high level of proliferation seen on the first day of culture (not shown, see reference 12), the rate for iron-containing media not containing transferrin dropped sharply on day 2 and remained low on day 3. Although the differences in the rate of thymidine incorporation within the series of ferric ion concentrations were very small, some trends were seen. On day 2, proliferation formed a slight peak at 20 μM ferric ion (Fig. 1 *B*). By day 3, however, increasing the ferric ion concentration decreased the already low levels of thymidine incorporation (Fig. 1 *C*).

The total DNA content of these whole kidney experiments paralleled the thymidine incorporation rates. On day 1, no consistent differences in the amount of DNA per kidney were observed (Fig. 1 *D*). After day 2, however, the explants in the transferrin-containing control culture contained more DNA than those cultured with only ferric chloride in the media. A slightly higher level of DNA content was found at 30 μM ferric ion concentration, but all differences were very small (Fig. 1 *E*).

Thymidine incorporation was also determined in transfilter experiments (Fig. 1 *F*). At 45 h in culture, those explants grown in medium containing 30 μM ferric ion had a slightly higher rate of proliferation than those cultured in medium containing 2 or 10 μM ferric chloride. However, the possible stimulatory effect of 30 μM ferric ion was clearly less than the effect of transferrin.

Histology revealed that explants cultured in IMEM (with 2 μM ferric ion) had no tubules (Fig. 2*a*), whereas abundant tubule formation occurred when transferrin was present (Fig. 2*b*). In the presence of 10 μM ferric ion, one or two poorly formed, small tubules were seen in three out of seven explants
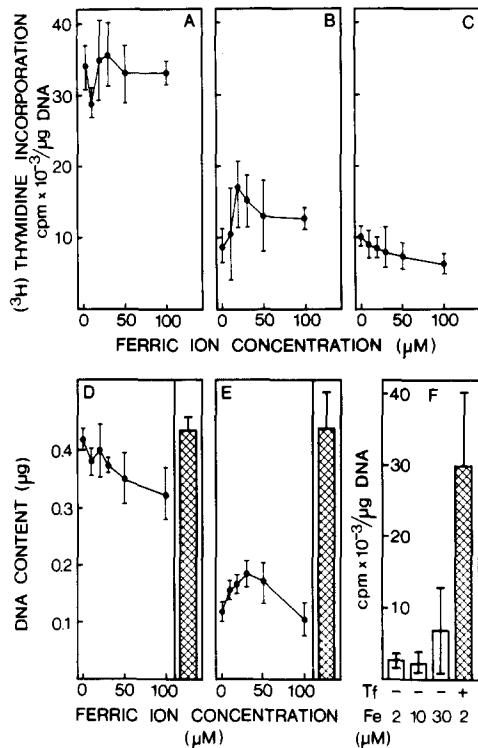
FIGURE 1 Lack of effect of free ferric chloride on thymidine incorporation in DNA of 11½-d-old whole kidneys (A–C) and 11-d-old metanephric mesenchymes cultured transfilter with an inducer tissue (F). (A) 11½-d kidneys on day 1. DNA synthesis is high in the chemically defined medium although no transferrin is present. The rate is not increased by increasing the concentration of medium iron. (B) 11½-d kidneys on day 2. A decrease in DNA synthesis has occurred when transferrin is not present. This sharp decrease cannot be overcome by adding iron. Only in explants grown in media containing iron near total serum concentration is there a very slight elevation in DNA synthesis. (C) 11½-d kidneys on day 3. DNA synthesis stays at a low rate, and increasing the iron content of the medium merely decreases the rate of thymidine incorporation. (D) DNA content of the 11½-d kidneys of Fig. A grown for 1 d. The bar to the right shows the DNA content when transferrin is present. (E) DNA content of the 11½-d kidneys of Fig. B grown for 2 d. The bar to the right shows the DNA content when transferrin is present. (F) 11-d-old metanephric mesenchymes placed on filters opposite spinal cord inducer tissue. On day 2, DNA synthesis in the mesenchyme is stimulated by transferrin (Tf), but not by ferric chloride (Fe). Only a small increase in DNA synthesis was seen with 30 µM iron. In the transfilter cultures, DNA synthesis was measured at 42–45 h as described (12). Results represent mean ± SD of three explants.

(Fig. 2c), but no other media supplemented with excess iron (20–50 µM) showed any tubulogenesis. Analysis of the response in whole-mount preparations confirmed that excess iron (10, 30, and 100 µM) was a very poor stimulator of tubulogenesis, since no tubules could be detected by this method (Table I). In contrast, explants grown in the presence of transferrin yielded a complete response; about 15 tubules per induced mesenchyme formed, so large a number that discerning the individual tubules was difficult.

Effect of FePIH

In order to determine the effect of iron independent of transferrin receptor interactions, we used the iron-chelator complex, FePIH, to transport iron across the cell membrane

(34). The rate of thymidine incorporation on the third day of culture in 11½-d kidney increased as the FePIH concentration increased up to 15 µM. This peak rate was only slightly less than the rate attained by kidneys cultured in the control media containing transferrin (Fig. 3A). When FePIH was present in the medium, the DNA content of the 11½-d kidneys increased almost as much as when transferrin was present (Fig. 3B).

The effect of FePIH was studied in transfilter experiments as well. Again, a dose-dependent increase of proliferation was observed (Fig. 3C). In explants cultured for 45 h in IMEM with 30 µM FePIH, thymidine incorporation was only slightly less than the rate of the explants cultured in IMEM with transferrin. Morphological examination from cross sections (Fig. 2d) and from whole mounts (Table I) of transfilter explants showed that FePIH promoted tubulogenesis. Only one out of 10 single mesenchyme transfilter explants grown in 2 µM FePIH had a few condensates, but all 10 explants cultured in 20 µM FePIH showed tubulogenesis, and there was an average of nine tubules per mesenchyme. The four explants grown in 30 µM FePIH had an average of 10 tubules per mesenchyme. The control mesenchymes cultured in the presence of transferrin had a response of 15 tubules per mesenchyme, so FePIH appeared to be ~60% as effective in promoting differentiation in these single mesenchyme experiments. The tubules that formed in response to FePIH (Fig. 2d) were very similar to those formed in response to transferrin (Fig. 2b). The mass of the explants was not crucial since results were similar when one or three mesenchymes were used (Table I).

It has been shown earlier than mesenchymes grown without inductor tissue do not become transferrin-dependent (12). Four such uninduced mesenchymes were cultured in the presence of 20 µM FePIH. None of these explants differentiated. Thus it is apparent that iron has no effect on uninduced mesenchymes.

Effect of Apotransferrin Versus Transferrin

Finally, we compared proliferation rates for tissues grown in media supplemented with apotransferrin and transferrin. The rate of DNA synthesis in 12-d kidneys was determined during a 3-d culture (Fig. 3A). After day 1, when all cultures have high levels of proliferation, transferrin was able to sustain a higher level of proliferation. Apotransferrin stimulated proliferation better than unsupplemented IMEM on day 3, but on day 2 the effect was only slight.

The effect of iron in the media was studied in the same series of experiments. The profile for apotransferrin-supplemented iron-free IMEM was similar to the profile for apotransferrin-supplemented IMEM. Transferrin-supplemented iron-free IMEM supported a profile of proliferation different from that of transferrin-containing IMEM. On day 3, the level of proliferation was the same as in the presence of apotransferrin, much lower than the proliferation rate of kidneys grown in IMEM supplemented with transferrin.

Also, in transfilter explants, clearly higher levels of proliferation were seen in kidneys grown in transferrin than in apotransferrin-containing media. Reintroducing iron to that batch of apotransferrin significantly increased the proliferation rate. Thus, the low proliferation observed for apotransferrin was not due to toxicity of the preparation. The iron content of the media did not influence the rate of thymidine incorporation observed (Fig. 4B).
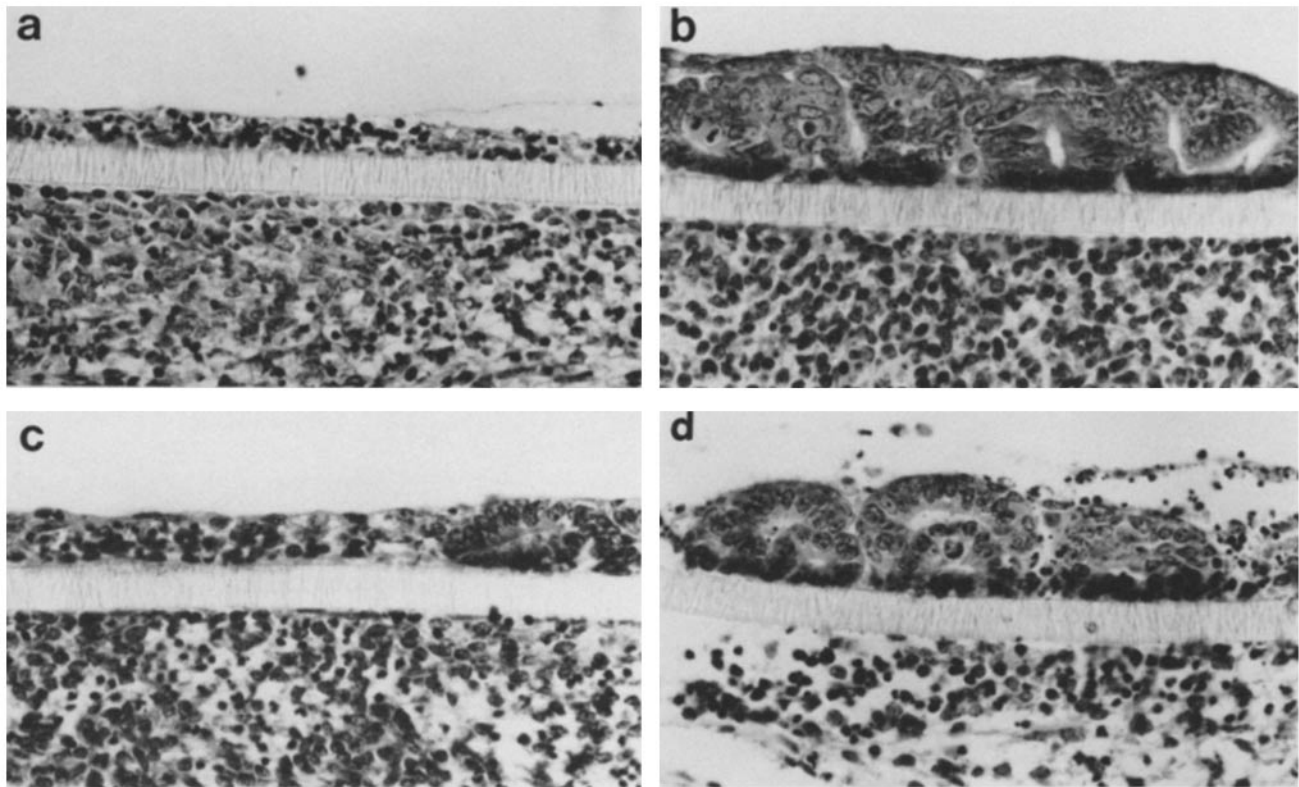
FIGURE 2 Light micrographs of cross sections of transfilter explants cultured for 3 d. (a) Explant cultured in unsupplemented IMEM. The undifferentiated mesenchyme is on top of the filter. (b) In explants cultured in IMEM supplemented with 50 µg/ml transferrin, extensive tubule formation is seen. (c) One of the few positive explants cultured in 10 µM ferric chloride. Note that only one small tubule has formed. (d) Extensive tubule formation is observed in all explants cultured in the presence of 30 µM FePIH. The tissue is nearly as well preserved as when transferrin is present. The filter thickness is 10 µm. × 550.

TABLE I

Morphological Response to FeCl₃ and FePIH in Transfilter Experiments

Supplement to MEM	Single mesenchyme experiments	Triple mesenchyme experiments
µM		
None	0/10	0/10
FeCl₃		
10	0/12	3/7
30	0/10	0/5
100	0/10	0/2
FePIH		
2	1/10	
20	10/10	
30	4/4	8/8
Transferrin (50 µg/ml)	10/10	10/10

Data are expressed as the number of explants positive for tubules per total number of explants. Tubule formation was determined by microscopic examination.

DISCUSSION

Previously, it was shown that the embryonic metanephric mesenchyme is dependent on two different types of exogenous stimuli for proliferation. The first is a close contact between the inducer tissue and the mesenchyme, but it can stimulate proliferation for only 1 d. Continued proliferation requires the presence of transferrin (12). Since inducer tissues do not produce transferrin, metanephric development is also regulated by tissues that produce transferrin. This suggests that both close cell contacts and humoral factors control proliferation of the metanephric mesenchyme. Since transferrin is an iron carrier (1, 2), we studied the role of iron in cell proliferation and differentiation of epithelial kidney tubules. It is shown here that the proliferation during subsequent stages is an iron-dependent event. Iron salts did not stimulate proliferation, but when iron was coupled to a lipophilic iron chelator a stimulation of proliferation was obtained.

Although some studies have shown that the addition of iron without transferrin stimulates cell proliferation (36) and differentiation (23), there are other well characterized systems in which nontransferrin iron is a poor stimulator (48) or cannot at all stimulate proliferation (45). We similarly found that the most effective iron concentration for differentiation of myoblasts (23) could not stimulate kidney differentiation. Although lower concentrations of ferric ion promoted slight tubule formation in a few cases, differentiation was poor and incomplete, and no stimulation of proliferation occurred.

It has been shown for other systems that transferrin supports proliferation better than apotransferrin (23, 31). We similarly found that iron-loaded transferrin is much better at promoting proliferation and supporting differentiation than pure apotransferrin and that the iron content of the media had only a secondary effect.

The kinetics of these experiments suggest the importance of using iron-saturated transferrin in chemically defined media for rapidly proliferating cells. Thus, it seems that once transferrin becomes iron depleted it can no longer support active proliferation. Apotransferrin binds iron slowly at neutral pH in the absence of weak chelators (4). It seems necessary
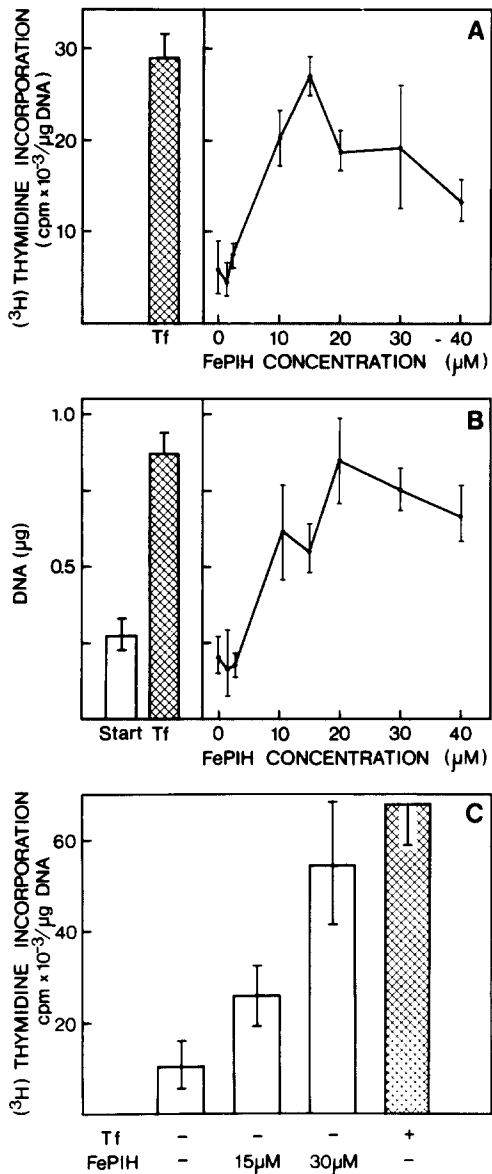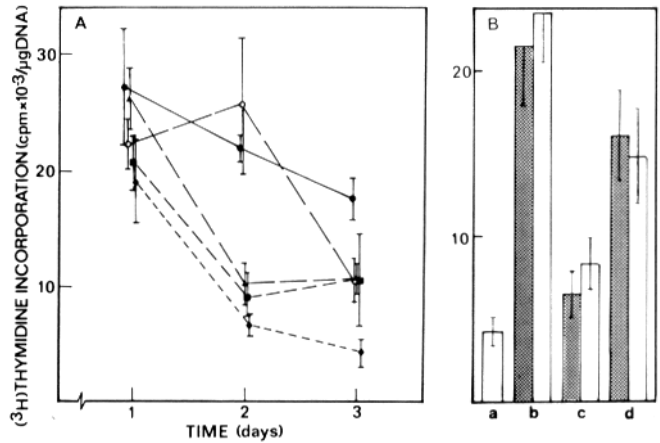
FIGURE 4 Role of iron-saturation in transferrin-dependent stimulation of thymidine incorporation in DNA in 12-d-old whole kidneys (A) and transfilter cultures (B). (A) Time course of thymidine incorporation in DNA of 12-d kidneys. ●, Transferrin (50 μg/ml) in medium containing 2 μM iron; ○, transferrin (50 μg/ml) in medium containing no iron; ▲, no transferrin, but medium containing 2 μM iron; ■, Apotransferrin (50 μg/ml) in medium containing 2 μM iron; ◆, Apotransferrin (50 μg/ml) in medium containing no iron. (B) Thymidine incorporation in DNA in mesenchymes cultured transfilter with inductor tissue, measured at 42–45 h (10). Shading indicates absence of free ferric ion in medium while open bars represent 2 μM ferric ions in medium. (a) IMEM not supplemented with any transferrin; (b) IMEM supplemented with transferrin; (c) IMEM supplemented with apotransferrin that was rendered iron-free by dialyzing the transferrin used in b against citrate; (d) IMEM supplemented with transferrin that was rendered iron-saturated from the apotransferrin used in b by dialyzing against ferric citrate in the presence of carbonate. Results are means ± SD of three explants.

FIGURE 3 Effect of FePIH on thymidine incorporation and DNA content of metanephric tissues. (A) 11½-d-old kidneys cultured in IMEM supplemented with 0–40 μM FePIH. In 15 μM FePIH, DNA synthesis in the kidneys is only slightly less than in kidneys grown in the presence of transferrin. The bar to the left shows DNA synthesis on day 3 of 11½-d-old kidneys cultured for 3 d in the presence of 50 μg/ml transferrin. (B) The DNA content of the explants of A grown for 3 d. In 15–30 μM FePIH, the DNA content has increased from the start value (left bar) to almost the same level as when transferrin (Tf) is present. (C) Thymidine incorporation of isolated 11-d-old mesenchyme induced transfilter spinal cord, cultured for 45 h in IMEM supplemented with 0–30 μM FePIH, or Tf. A dose-dependent response to FePIH is seen, and 30 μM FePIH stimulated DNA synthesis only slightly less than transferrin. Results are mean ± SD of three explants.

then to use the holoprotein in cultures with chemically defined media where proliferation or differentiation occurs at a rapid rate and where cells require large amounts of iron rapidly. In all cultures, weak chelators of iron, e.g., citrate or ascorbate, could improve the association rate of free iron and apotransferrin in the media.

For several reasons, neither studies with free ferric ion nor comparisons of apotransferrin with transferrin tell us whether

some direct receptor-transferrin interactions have important effects on proliferation. The inadequacy of iron salts to increase proliferation is most likely due to the inability of the cells to incorporate free ferric ion which is normally not found physiologically. If this is the case, negative results are to be expected, since neither the receptor nor intracellular iron levels are affected. Further, it has been shown that the binding of apotransferrin to the receptor at neutral pH is much less stable than that of transferrin (8, 24, 25). Attempts to assay receptor-mediated proliferation signals in the absence of iron are thus confounded by the unequal affinity constants for the holoprotein and the apoprotein. Hence, the finding that apotransferrin is less effective as a stimulator of cell proliferation does not necessarily prove that the iron ion is the stimulator of proliferation. The fact that metal chelators such as picolinic acid (14) and desferroxamine (31, 49) interfere with proliferation suggests that the iron in transferrin is crucial, but again the evidence is indirect since those chelators inhibit proliferation and toxic effects can not be excluded.

Novel approaches to this problem are therefore needed. One was recently suggested by Block and Bothwell (6), who provided nutritive iron to cells via ferritin-bound antibodies to specific cell-surface markers. Evidently, endocytosed marker-antibody-ferritin complexes can provide iron intracellularly which sustains cell proliferation. Our attempt to bypass the transferrin receptor complex depended on the iron-binding capacity of the lipophilic PIH. Earlier reports show that this compound can remove iron from and provide iron to reticulocytes (32–34). We reasoned that these properties of

PIH could be used in our system to positively influence proliferation.

It was found that FePIH at concentrations near total serum levels of ferric ion can sustain proliferation only slightly below that of transferrin. The model system used is fortuitous in that the replacement of transferrin by FePIH can be followed by both proliferation and cell differentiation. Epithelial differentiation was clearly visible in mesenchymes cultured with FePIH. FePIH does not merely permit cell growth; it supports large shifts in cell type and polarization and organization of the cells into a differentiated tissue, events that are correlated with proliferation in this system (12). Therefore, it can be concluded that the response to FePIH was physiologic and, further, that the role of transferrin in the proliferation of metanephric cells is simply to deliver iron intracellularly.

The chemical features of PIH may explain why it can stimulate proliferation and differentiation as effectively as transferrin. In most systems, free iron cannot enter the cells directly but must be delivered by transferrin through the receptor-mediated endocytosis pathway (8, 25, 30, 47). This provides a means for the cells to control their proliferation. PIH, however, is lipophilic, and seems to traverse the cell membrane directly (32–34). Other iron chelators apparently do not have such properties since they inhibit (14, 29, 31) rather than stimulate cell proliferation. Our study suggests that FePIH could be used to investigate the role of iron in other systems in which transferrin is important for cell replication. It is now widely thought that the transferrin receptor density is important for the control of cell proliferation (10, 17, 40, 41, 46). Use of the FePIH complex, which bypasses the receptor-mediated route of iron uptake, makes it possible to study these problems experimentally.

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