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Lipopolysaccharide Potentiates the Effect of Hepatocyte Growth Factor upon Replication in Lung, Thyroid, Spleen, and Colon in Rats *in Vivo*

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Induction of replication may potentiate *in vivo* gene therapy, as some viral vectors only transduce dividing cells. Hepatocyte growth factor (HGF) increases the percentage of replicating hepatocytes to 18-fold that in normal rats, and lipopolysaccharide (LPS) modestly potentiates this effect. In this study, the effect of iv HGF upon replication in other organs was determined. HGF at 10 mg/kg resulted in replication that was ≤3-fold that of normal rats in alveolar and proximal renal tubular cells. HGF alone had no effect upon replication of epithelial cells from the bronchi, thyroid, pancreas, or colon or upon cells from the muscle, pancreatic islets, spleen, blood vessels, or thymus. HGF and LPS at 5 mg/kg resulted in replication that was 9-fold that of normal rats in alveolar cells, 25-fold in bronchial epithelial cells, 4-fold in thyroid epithelial cells, 1.5-fold in the red pulp of the spleen, and 2-fold in colonic epithelial cells. The synergistic effect may be due to the fact that LPS upregulated the HGF receptor c-met in thyroid, spleen, and colon. We conclude that iv administration of HGF alone is relatively specific for inducing hepatocyte replication and would allow selective gene transfer into the liver.

Key Words: **bromodeoxyuridine; labeling index; gene therapy; retroviral vector.**

INTRODUCTION

Hepatocyte growth factor (HGF) is a 90-kDa heterodimeric protein that binds to the c-met receptor on the surface of cells and induces mitogenesis, morphogenesis, and/or motogenesis (1). Although initially isolated as a factor that induces replication of hepatocytes, HGF can also induce replication of a variety of other cell types. It is identical to scatter factor, which was isolated by virtue of its ability to cause Madin–Darby canine kidney (MDCK) cells to disaggregate or scatter. In addition, HGF can induce tubule formation in endothelial cells in culture. The signaling pathways responsible for these effects include the activation of p21-Ras and its downstream mediator extracellular signal-regulated kinase [ERK; also known as mitogen-activated protein kinase (MAPK)], phosphatidylinositol 3 kinase (PI3K), phospholipase C γ (PLC-γ), and $pp60^{c\text{-}src}$ (2–4).

We have been using HGF in rats to induce replication of hepatocytes in order to facilitate gene therapy with retroviral vectors that only transduce dividing cells. Multiple doses of HGF given over a 24-h period for a cumulative dose of 10 mg/kg induced replication in 11.1% of hepatocytes at 24–33 h after the first dose of HGF, and 8% of cells at 33–42 h (5). Furthermore, administration of a low and overtly nontoxic dose (5 mg/kg) of lipopolysaccharide (LPS) in addition to this dose of HGF increased the percentage of replicating hepatocytes to 18.4% at 33–42 h, although it had no effect upon replication at 24–33 h. The potentiating effect of LPS was even more apparent when a 10-fold lower of dose of HGF was used, as LPS increased replication at 33–42 h from 2.1 to 9.0%. The latter finding was consistent with another study in which tumor necrosis factor α (TNFα) or LPS potentiated the effect of low dose HGF upon replication of hepatocytes in rats by 4-fold (6).

In this study, the effect of systemic HGF with or without LPS upon replication of cells in other organs was examined for two reasons. First, we are interested in any possible adverse effects upon other organs that might occur when HGF is being used to facilitate hepatocyte transduction, such as replication and transduction of other cell types. Second, iv HGF might be used to induce

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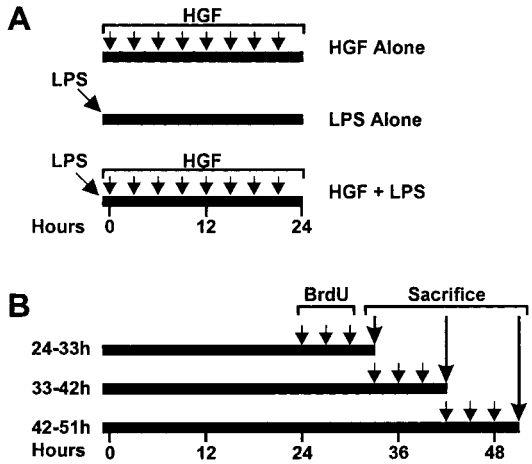


FIG. 1. Schematic diagram of the injection regimen for HGF, LPS, or both, and the method for BrdU labeling. (A) Injection regimen. For rats that received HGF alone, HGF was injected iv every 3 h at 1.25 mg/kg/dose beginning at time = 0 h, and was continued every 3 h for a total of 8 doses. The cumulative dose of HGF was 10 mg/kg, and PBS was injected at −1 h. For rats that received LPS alone, LPS was injected ip at −1 h and PBS was injected at other times. For rats that received HGF and LPS, LPS was injected at −1 h, and HGF was injected every 3 h at 0 to 21 h. (B) BrdU labeling protocol. For determination of the percentage of cells that replicated between 24 and 33 h, 100 mg/kg of BrdU was injected at 24, 27, and 30 h, and the animal was sacrificed at 33 h. Frozen sections were analyzed for the percentage of replicating cells with anti-BrdU immunostaining. A similar strategy was used to determine the percentage of cells that replicated at 33–42 h or 42–51 h after the first dose of HGF.

regeneration and/or gene transfer in other organs that respond to HGF *in vivo*. Cell types in addition to the hepatocyte (7) that have been reported to replicate in response to HGF in culture and/or *in vivo* include alveolar cells (8–11), bronchial epithelial cells (11, 12), proximal tubular cells (13–18) and glomerular cells of the kidney (19, 20), intestinal epithelial cells (21, 22), thyroid epithelial cells (23–25), pancreatic epithelial (26, 27) and endocrine (28, 29) cells, skeletal muscle cells (30–33), monocytes (34), and endothelial cells (35). In most cases, the effect of HGF has not been determined *in vivo*, or HGF was only administered locally due to the limited availability of HGF. We therefore tested the effect of HGF upon replication of other cell types after iv administration. We find that HGF alone has no or only a modest effect upon replication in organs other than the liver, and that this would likely result in selective gene transfer into the liver when using a viral vector that only transduces replicating cells. LPS potentiated the effect of HGF upon inducing replication in the lung, colon, thyroid, and spleen. HGF in combination with LPS, or possibly some downstream mediators of the acute phase response, might be used to augment gene transfer into other organs.

MATERIALS AND METHODS

Purification of HGF protein. Human HGF was produced by 293-N3S cells (Microbix Biosystems, Toronto, Ontario) that were infected with the adenoviral vector Ad.CMV.HGF (36) and purified over a heparin Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) followed by an SP-Sepharose (Pharmacia) column, as described previously (5). The HGF protein level was determined by ELISA, and the bioactivity was determined by scatter assay of MDCK cells as described (5). HGF standards were obtained from Becton–Dickinson Labware (Bedford, MA). There was good correlation between the HGF protein levels and the bioactivity.

Animal procedures. All animals received humane care according to the criteria outlined in *The Guide for Care and Use of Laboratory Animals*. Male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis IN) weighing 220 to 260 gm were anesthetized with metofane (Mallinckrodt Veterinary, Inc., Mundelein, IL), and HGF (1.25 mg/kg in ~1 ml per dose) was injected into the penile vein with a 30-gauge needle as previously described (5). LPS from *Escherichia coli* serotype 0111:B4 (Sigma Chemical, St. Louis, MO) was diluted in pyrogen-free normal saline and injected ip at 5 mg/kg. For determination of the labeling index, 3 doses of 100 mg/kg of 5-bromo-2′-deoxyuridine (BrdU) per dose were injected ip every 3 h, the animal was sacrificed 3 h after the last dose, and the organs were frozen in optimal cutting temperature (OCT) compound (Bayer Corp., Mishiwaka IN). Lungs were injected with a solution containing 50% PBS:50% OCT prior to harvest.

BrdU immunostaining and quantitation of the labeling index. BrdU immunostaining was performed on 8-μm frozen sections of organs. Either a goat anti-BrdU antibody generously provided by Steve Cohn (37) or a commercially available sheep anti-BrdU antibody (Catalog No. PAB105, Maine Biotechnology Services, Inc., Portland ME, dilution 1:400) was used as described in detail (38). Slides were incubated with a horse-radish peroxidase (HRP)-coupled anti-goat/sheep IgG at a 1:200 dilution, and the brown color developed with 3,3′-diaminobenzidine.

For all organs, the labeling index was determined by counting the total number of BrdU-labeled cells of a particular phenotype and dividing by the total number of cells of that phenotype, as determined by hematoxylin and eosin (H & E) staining. For most cell types, 20 different randomly chosen high-power (40×) fields were evaluated. Alveolar cells were identified with the help of a pathologist by their presence at the surface of the alveolus. Bronchial epithelial cells were identified by their presence within a circular structure in which the nuclei were aligned in a columnar epithelium, and all bronchi from one or more sections were evaluated. For the large intestine, only cells within the epithelium of the crypt were evaluated, and analysis was limited to crypts that extended from the base to the lumen of the colon and contained nuclei that lined up well throughout (39). In addition, the height of the crypt from the base of the epithelium to the luminal surface was determined with a micrometer. In the kidney, proximal tubular cells were identified by their presence within a circular structure within a region of the slide that contained an adjacent glomerulus (16). In the thyroid and pancreas, epithelial cells were similarly identified by their presence within a circular structure. For pancreatic islets, the number of labeled cells was divided by the total number of cells in each islet. In the spleen, the red and the white pulp were identified by H & E staining, and the percentage of replicating cells within each region was determined separately. In the thymus, the medulla and the cortex were identified by H & E staining, and the percentage of replicating cells in each region was determined separately. In the skeletal and cardiac muscle, all nuclei within the body of the muscle were evaluated.

Immunoblot for c-met. Organs were homogenized with 400 μl of RIPA buffer (1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, with 0.5 mM PMSF, and 2 μg per milliliter each of aprotinin, antipain, and leupeptin (40)] per 100 mg of tissue and centrifuged at 4°C in a microfuge at 13,000*g* for 20 min. The protein concentration in the supernatant was determined by Bradford assay. Samples were electrophoresed on an 8% reducing SDS–PAGE gel using 50 μg per sample for spleen and liver, 100 μg for thyroid, and 75 μg for large intestine. The gel was transferred to an Immobilon-P membrane from Millipore Corp. (Bedford MA), and incubated with blocking buffer (TBS with 5% fat-free milk and 0.1% Tween 20) for 1 h at RT. The membrane was incubated overnight in blocking buffer with a 1:200 dilution of a rabbit anti-c-met antibody No. SP260 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then incubated for 1 h at RT with an HRP-conjugated anti-rabbit antibody at a 1:5000 dilution in blocking buffer.

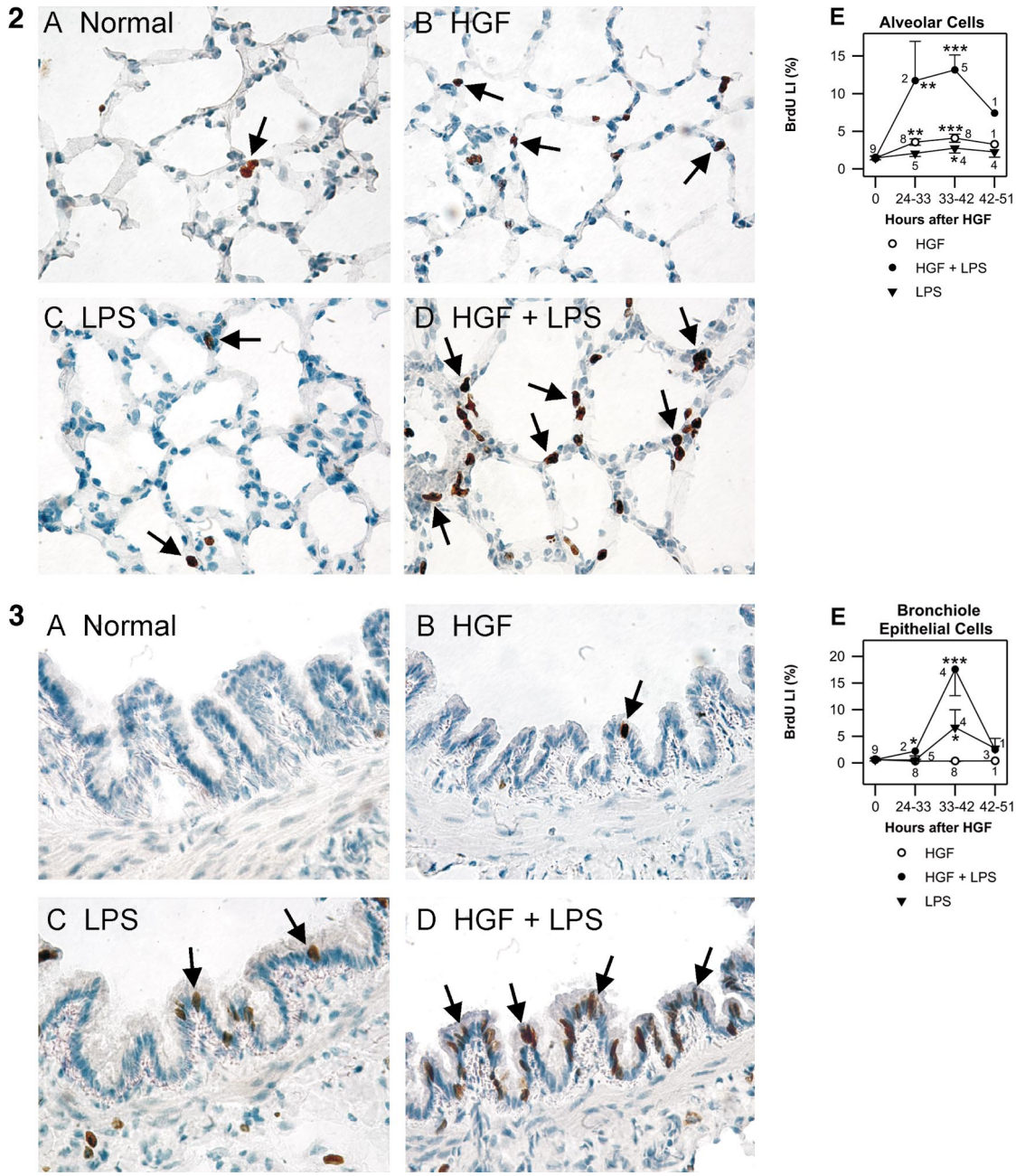


FIG. 2. (A–D) Examples of replication in the alveoli. Rats received no treatment (A) or were treated with HGF alone (B), LPS alone (C), or both HGF and LPS (D) according to the regimen shown in Fig. 1. The labeling index was determined at 33 to 42 h after time 0, as detailed in Fig. 1. Frozen sections underwent anti-BrdU immunostaining with a development procedure that results in a brown shading and were stained with H & E. The black arrows identify nuclei of cells that had recently replicated, while cells with blue nuclei had not replicated. All photographs were at a 60× original magnification. (E) Quantitation of the BrdU labeling index in the alveoli. The average ± SEM of the BrdU labeling index (LI) in the alveolar cells for the indicated number of animals is shown. Values that were significantly different from those in normal rats using Student's *t* test are identified with asterisks; the criteria for significance are detailed under Materials and Methods. Values in normal rats are shown as the 0-h data point.

FIG. 3. (A–D) Examples of replication in the bronchial epithelium. Rats were treated as noted in the legend to Fig. 2, and anti-BrdU followed by H & E staining was performed to determine the percentage of replicating cells in the bronchial epithelium. Rats received no treatment (A) or were treated with HGF alone (B), LPS alone (C), or both HGF and LPS (D). All photographs were at a 60× original magnification. The black arrows identify BrdU-labeled bronchial epithelial cell. The lumen of the bronchus is at the top for all panels. (E) Quantitation of the BrdU labeling index in bronchial epithelial cells.

Samples were developed with chemiluminescence using Lumi-light Western blotting substrate (Boehringer Mannheim Corp., Indianapolis, IN). Protein levels were determined by densitometry of the autoradiogram, and normalized to the level found in normal liver after the subtraction of the background signal.

Statistical analyses. Statistical analyses between two groups of animals were performed with the program Instat from GraphPAD Software (San Diego, CA) using Student's *t* test. * indicates a *P* value between 0.05 and 0.005, ** indicates a *P* value between 0.005 and 0.0005, and *** indicates a *P* value <0.0005.

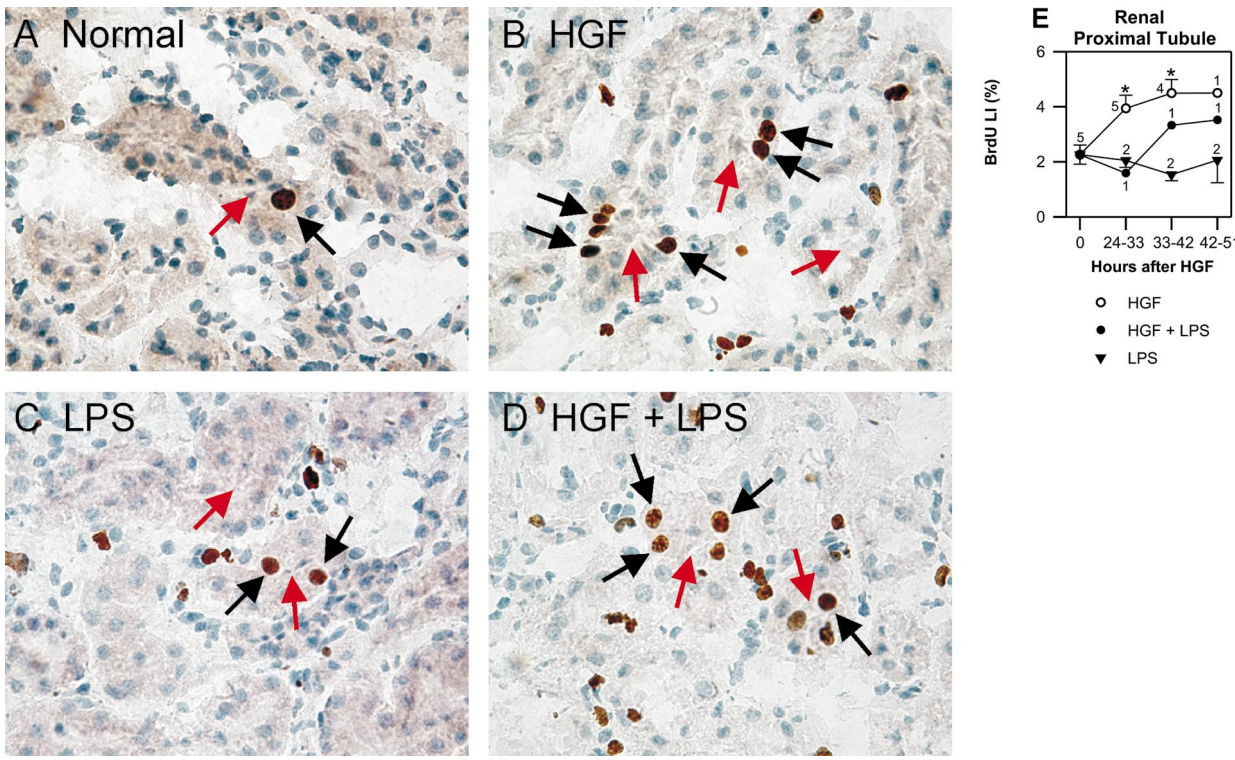


FIG. 4. (A–D) Examples of replication in the proximal tubular cells of the kidney. Rats were treated as noted in the legend to Fig. 2, and anti-BrdU followed by H & E staining was performed to determine the percentage of replicating cells in the proximal tubular cells of the kidney at 33 to 42 h after time 0. Rats received no treatment (A) or were treated with HGF alone (B), LPS alone (C), or both HGF and LPS (D). All photographs were at a 60× original magnification. BrdU-labeled proximal tubular cells are identified with a black arrow. Red arrows identify the lumen of the tubule. (E) Quantitation of the BrdU labeling index in proximal tubular cells of the kidney.

RESULTS

HGF alone, LPS alone, or HGF and LPS were administered to rats as diagramed in Fig. 1. For animals that received HGF alone, HGF was injected iv as 8 doses over 24 h for a cumulative dose of 10 mg/kg, with the time of the first dose designated as 0 h. In addition, PBS was injected ip at −1 h as a control for the nonspecific effect of an ip injection at this time. For animals that received LPS alone, LPS was injected as a single dose ip at the time designated −1 h, and PBS was injected iv at times when the other animals received HGF. Some animals (HGF and LPS) received both HGF and LPS at the indicated times. All animals received three doses of BrdU over a 6-h period, and were sacrificed at 3 h after the last dose. Since BrdU gets incorporated into the nuclei of cells that are replicating their DNA, this allows identification of the cells that replicated over a 9-h window of time. For all organs, two controls were injected with PBS at times when other animals received HGF and/or LPS, and the BrdU labeling index was determined over the 9-h window from 24–33 h, and 33–42 h. Since the values in these rats resembled those from normal rats that did not receive any injections of PBS and were evaluated for BrdU-labeled cells over a 9-h window of time, values from normals that did not receive any PBS injections were pooled with those that did.

Lung Alveolar Cells

HGF alone had a modest effect upon replication of alveolar cells, as it increased the percentage of replicating cells from 1.4% in normal rats (Fig. 2A) to 3.6 ± 0.4% at 24–33 h ($P = 0.0005$ vs normal) and 4.1 ± 0.5% at 33–42 h (Fig. 2B; $P = 0.0003$ vs normal). Most replicating cells were cuboidal and were located within at the corners of alveoli, and thus appeared to be primarily type II pneumocytes. LPS alone had a modest effect upon replication at 33–42 h, when 2.75 ± 0.6% of alveolar cells were replicating (Fig. 2C; $P = 0.03$ vs normal). This was likely a compensatory response to LPS-induced lung damage (41). The effects of HGF and LPS were synergistic, as the combination of HGF and LPS resulted in replication in 11.7 ± 5.2% of alveolar cells at 24–33 h ($P = 0.007$ vs HGF alone and $P = 0.02$ vs LPS alone) and replication of 13.1 ± 2% of cells at 33–42 h (Fig. 2D; $P = 0.0002$ vs HGF and $P = 0.03$ vs LPS alone). Many, but not all, of the replicating cells had the morphological appearance of type II pneumocytes. We conclude that HGF alone increased replication of alveolar cells to 2.9-fold that of normal rats, while HGF and LPS increased replication to 9.4-fold that of normal.

Bronchial Epithelial Cells

In the normal rat (Fig. 3A), only 0.6 ± 0.2% of bronchial epithelial cells were replicating, and HGF alone (Fig. 3B) had no effect upon the percentage of replicating cells at any of the time points. The combination of HGF and LPS (Fig. 3D) resulted in replication of 17.6 ± 4.9% of cells at 33–42 h. This was significantly higher than the amount of replication observed in normal animals ($P = 0.0002$), but was not significantly different from the value of 6.6% ± 3.3% observed in LPS-treated rats (Fig. 3C) at the same time point. The percentage of replicating bronchial epithelial cells in the LPS-treated rats was significantly higher than in normal animals ($P = 0.006$), which may be a compensatory response to lung damage (41). We conclude that HGF alone has no effect upon replication of bronchial epithelial cells. The combination of LPS and HGF resulted in 29-fold as many replicating cells as were present in normal rats, and LPS alone was responsible for at least part of this effect.

Kidney

In the normal kidney (Fig. 4A), 2.3 ± 0.3% of proximal tubular cells replicated during a 9-h interval, and this was increased to 1.7-fold that of normal at 24–33 h in response to HGF ($P = 0.02$ vs normal), and to 2-fold that of normal at 33–42 h (Fig. 4B; $P = 0.007$ vs normal). LPS alone (Fig. 4C) had no effect upon the percentage of proximal tubular cells that were replicating, and the combination of HGF and LPS (Fig. 4D) did not increase the percentage of replicating cells compared with that observed with HGF alone in a limited number of animals. We conclude that HGF alone has a modest effect upon replication of proximal tubule cells, and that LPS does not potentiate this effect.

Large Intestine

For the large intestine, each crypt was quantitated from the values obtained directly from a 2-dimensional section. Normal rats contained 86.8 ± 2.5 total cells per crypt, 22.3 ± 2.9 replicating cells per crypt, and an average labeling index over a 9-h period of 23.0 ± 3.0% (Fig. 5A). In addition, the BrdU-labeled epithelial cells were located primarily in the lower 40% of the crypt. Neither HGF alone (Fig. 5B) nor LPS alone (Fig. 5C) had a significant effect upon the percentage of colonic epithelial cells that were replicating at any of the time intervals. In contrast, the combination of HGF and LPS resulted in a labeling index that was 1.7-fold that of normal intestine at 24–33 h ($P = 0.03$ vs normal), and 2.1-fold that of normal at 33–42 h (Fig. 5D; $P = 0.001$ vs normal). This was due to the fact that the region in which labeling occurred extended to include the lower 60% of the crypt, and a higher percentage of cells were replicating in regions that contained labeled cells.

An increase in the number of replicating cells in animals that received HGF and LPS might result in a taller crypt due to more cells per crypt if the survival time remained constant. However, although the crypts were ≈8% taller at 33–42 and 42–51 h after administration of HGF and LPS than were crypts from normal animals (Fig. 5F), these differences were not statistically significant. A similar but statistically insignificant increase was observed in the number of cells per crypt for these animals (data not shown). Thus, the increase in the percentage of replicating cells did not result in taller crypts.

Thyroid

In normal rats (Fig. 6A), 1.6 ± 0.4% of the thyroid epithelial cells were replicating. Neither HGF alone (Fig. 6B) nor LPS alone (Fig. 6C) had any effect upon the percentage of replicating cells. However, the combination of HGF and LPS resulted in replication in 6.7% of thyroid epithelial cells (Fig. 6D; $P = 0.001$ vs normal). We conclude that HGF and LPS resulted in 4.3-fold as many replicating thyroid epithelial cells as in normal rats.

Spleen

In the normal spleen (Fig. 7A), 26.3 ± 1.6% of cells in the red pulp had replicated over a 9-h window of time. Administration of HGF alone (Fig. 7C) had no effect, while the administration of LPS alone (Fig. 7E) resulted in a modest and statistically insignificant increase in the percentage of replicating cells. In contrast, the administration of HGF and LPS resulted in replication of 39.1% of the cells at 33–42 h (Fig. 7G), which was statistically higher than in the other groups ($P = 0.0009$ vs normal rats, $P = 0.0006$ vs HGF alone, and $P = 0.04$ vs LPS alone). In the white pulp, only 5.2 ± 0.5% of the cells were replicating in the normal rat. HGF alone resulted in a modest and statistically insignificant increase in the percentage of replicating cells. Administration of LPS alone resulted in a marked increase in the percentage of replicating cells to >16.9% at all three time intervals ($P < 0.0001$ vs normal). This may be due to the known mitogenic effect of LPS upon B cells (42). The combination of HGF and LPS resulted in replication of >25.5% of all cells at all time intervals ($P < 0.0001$ vs normal), although this was not significantly higher than the levels observed in rats that received LPS alone. We conclude that LPS alone resulted in 5.2-fold as many replicating cells in the white pulp as in normal rats, while the combination of HGF and LPS resulted in 1.5-fold as many replicating cells in the red pulp as in normals.

Thymus

In the cortex of the normal thymus (Fig. 8A), 13.4 ± 0.5% of cells were replicating. Although HGF alone did not have an effect upon the percentage of replicating cells in the cortex (Fig. 8B), there were only 10.2% as many replicating cells at 33–42 h after LPS as were present in normal animals ($P = 0.004$ vs normal), and only 12.2% as many at 42–51 h (Fig. 8C; $P = 0.0005$ vs normal). The combination of HGF and LPS also reduced the percentage of replicating cells in the cortex (Fig. 8D). In the medulla

of the thymus, the administration of HGF alone, LPS alone, or both HGF and LPS had no effect upon the percentage of replicating cells. We conclude that LPS results in a dramatic reduction in the percentage of replicating cells in the cortex of the thymus.

Determination of c-met Protein Levels

A possible explanation for the synergistic effect of HGF and LPS upon replication in some organs was that LPS induced expression of the HGF receptor c-met. We therefore tested the effect of LPS upon c-met protein levels, as shown in Fig. 9. c-met was readily detectable in normal liver, and LPS had no effect upon the levels (data not shown). LPS resulted in c-met levels that were 4.1-fold that of normal in the thyroid ($P = 0.02$) and 3.9-fold that of normal in the spleen ($P = 0.04$) at 42 h. In the intestine, LPS resulted in c-met levels that were 4.6-fold that of normal at 9.5 h ($P = 0.02$), and 6.5-fold that of normal at 42 h ($P = 0.002$). c-met was not detectable in the lung at 33 or 42 h after the administration of LPS (data not shown). We conclude that LPS induces expression of c-met in thyroid, spleen, and intestines.

DISCUSSION

It was previously demonstrated that HGF increased the percentage of replicating hepatocytes, and that the administration of LPS in combination with HGF augmented this effect (5). This study was undertaken to determine the effect of HGF with or without LPS upon replication in other organs that have been reported to respond to HGF *in vitro.* The response of any individual cell type to the iv administration of HGF will be a function of (i) the delivery of HGF to that cell; (ii) the levels of the HGF receptor c-met in that cell; and (iii) the intrinsic ability of that cell to exhibit a mitogenic response to HGF. The robust increase in replication of hepatocytes after iv injection of HGF may be due to the fact that the liver is the major site of uptake of HGF after an iv injection, as it contains 29% of the injected dose at 15 min (43–45). The liver also has very high levels of c-met (46–48), and thus is poised to respond rapidly to HGF. Finally, hepatocytes are highly responsive to HGF *in vitro,* with a 10-fold increase in the percentage of replicating cells with a half-maximal effect at 3 ng/ml, and a maximal effect at 10 ng/ml (7). Table 1 shows the levels of the c-met receptor and the efficiency of uptake of HGF in different organs. It also summarizes the ability of a particular cell type to exhibit a mitogenic response to HGF and the concentration required for a maximal effect. Finally, this table shows the effect of HGF or HGF with LPS upon replication of different cell types in this study.

Lung

In this study, alveolar cells of rats that were treated with HGF alone had 2.9-fold as many replicating cells as did normal rats. This effect is consistent with the fact that lung takes up low to moderate amounts of HGF after iv injection, contains low to moderate levels of c-met, and the ability of alveolar cells to respond to HGF *in vitro* and *in vivo.* The administration of LPS augmented the effect of HGF by 3.2-fold, resulting in replication of 9.4-fold as many cells as in normal rats. This may be due to upregulation of the c-met receptor in response to LPS, as LPS induces interferon γ (IFNγ) expression (49), and expression of c-met by alveolar cells is dramatically upregulated by IFNγ *in vivo* (50). In addition, several other cytokines that are induced by LPS increased c-met expression in a variety of transformed cell lines (51). Although this study did not detect an increase in c-met protein levels in lung at 33 or 42 h after the administration of LPS, it remains possible that c-met was induced earlier and levels had returned to normal by the time of analysis. A similar potentiation of replication in response to HGF and LPS was observed in bronchial epithelial cells, where HGF alone had no effect, but LPS with HGF resulted in replication of 29-fold as many cells as in normal rats.

After administration of HGF and LPS, the overall percentage of replicating cells was 11.7% for alveolar cells and 17.6% for bronchial epithelial cells. This should be sufficient to achieve a moderate to high degree of gene transfer provided that the vector can be administered at a sufficient dose in a way that it has assess to these cells. We are currently testing if LPS induces c-met expression at earlier times in alveolar and bronchial epithelial cells *in vivo* and if IFNγ is sufficient for this effect. If it is, the combination of HGF and IFNγ might be used to achieve optimal induction of replication in the lung for gene therapy with less toxicity.

Kidney

HGF resulted in 2-fold more replicating cells in the proximal tubule cells of the kidney than were present in normal rats. This response is consistent with the fact that the kidney takes up large amounts of HGF after iv injection and contains high levels of c-met. The relatively modest effect of HGF upon replication in proximal tubular cells *in vivo* in this study may be due to the fact that the replicative response *in vitro* is modest and requires high concentrations of HGF. In contrast to the lung, the combination of HGF and LPS did not further increase the percentage of replicating cells over that observed with HGF alone. This may reflect the fact that c-met levels are already very high in the kidney, or may be due to other mechanisms. In this study, the peak effect of HGF resulted in replication of 4.5% of all proximal tubular epithelial cells *in vivo.* This might make it possible to transfer a gene into a low to moderate number of these cells with vectors that only transduce dividing cells. The overall percentage of cells that were replicating in the glomeruli was 3.6-fold that of normal in response to HGF alone, and LPS did not augment this effect (data not shown). As the poor histology of frozen sections precluded identification of the

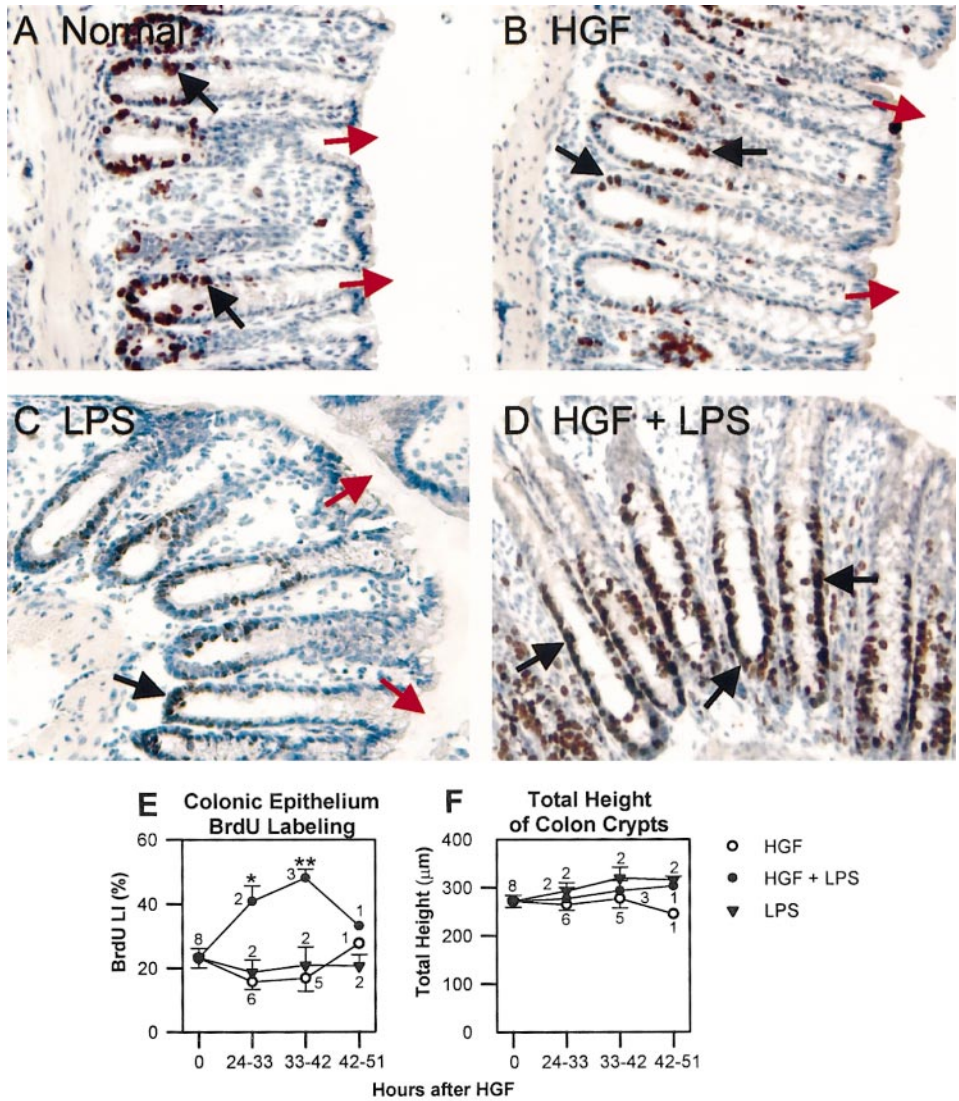


FIG. 5. (A–D) Examples of replication in epithelial cells of the colon. Rats were treated as noted in the legend to Fig. 2, and anti-BrdU followed by H & E staining was performed to determine the percentage of replicating epithelial cells of the colon at 33 to 42 h after time 0. Rats received no treatment (A) or were treated with HGF alone (B), LPS alone (C), or both HGF and LPS (D). All photographs were at a 30× original magnification. BrdU-labeled colonic epithelial cells are identified with a black arrow. Red arrows identify the lumen of the colon. (E) Quantitation of the BrdU labeling index in colonic epithelial cells. (F) Quantitation of the height of the crypts of the colon. Crypt heights were measured from the base of the crypt to the lumen of the colon and plotted as the average ± SEM in micrometers. There were no significant differences between samples from normal rats and those from any of the experimental groups.

cell types that were labeled, additional studies are currently being performed using immunocytochemistry to identify the specific cell types.

Large Intestine

In this study, HGF alone had no effect upon replication of colonic epithelial cells, although the combination of HGF and LPS resulted in 2.1-fold as many replicating cells as were found in normal rats. The lack of a response to HGF alone may reflect the fact that there are only moderate to low amounts of c-met in the colon, the delivery of HGF to the colon is low after iv injection, and/or intestinal epithelial cells require very high doses of HGF and have only a very modest replicative response to HGF *in vitro*. The mechanism by which LPS increased the responsiveness to HGF was likely due at least in part to upregulation of c-met, as LPS resulted in a 4.6- to 6.5-fold increase in levels of c-met in the large intestine.

Since epithelial cells of the crypts survive for 3 to 8 days (52), the increase in the percentage of replicating cells from 23.1% in normal rats to 48.1% in response to HGF and LPS might be expected to increase the height of the crypt. However, a statistically significant increase in the number of cells in the crypt or the height of the crypt was not observed. This suggests that cell death may be determined by the position of a cell within the crypt rather

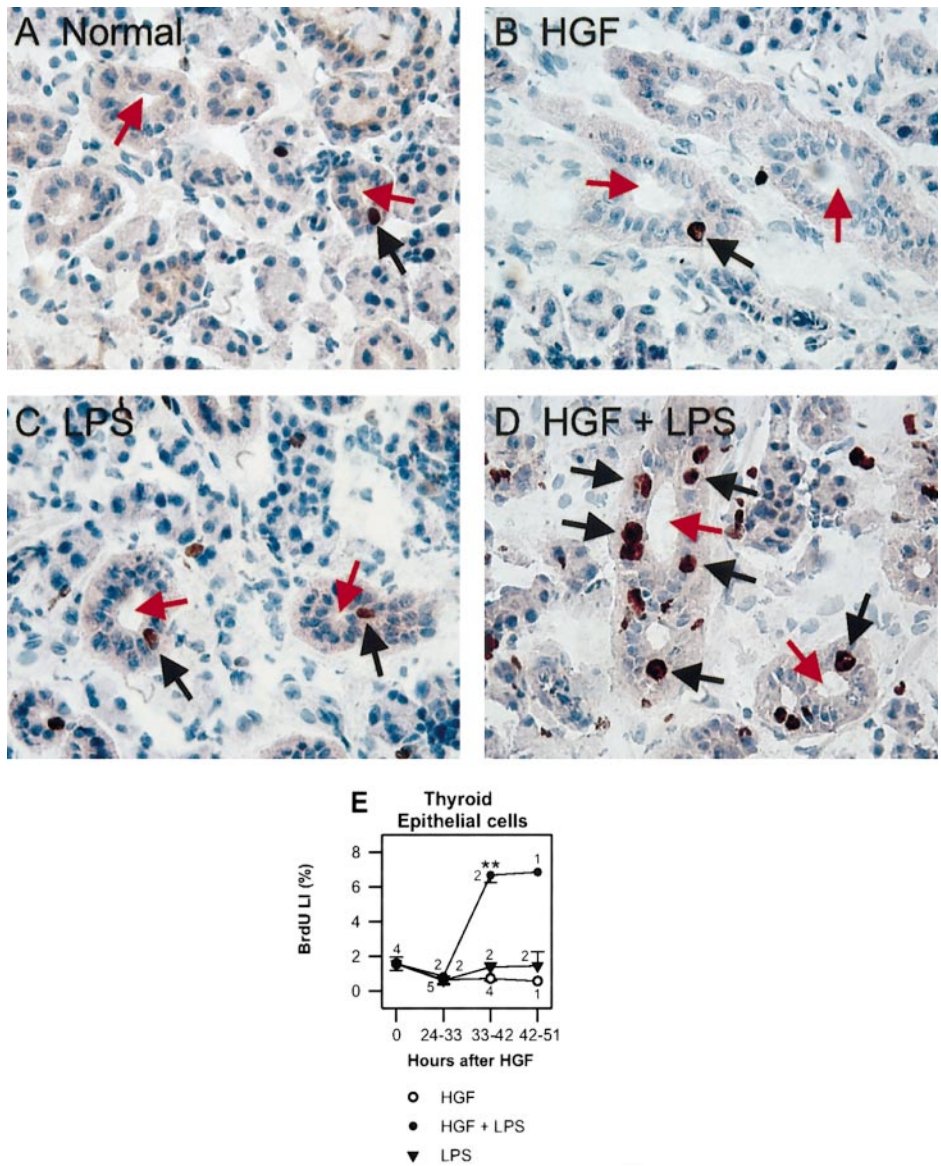


FIG. 6. (A–D) Examples of replication in the epithelial cells of the thyroid. Rats were treated as noted in the legend to Fig. 2, and anti-BrdU followed by H & E staining was performed to determine the percentage of replicating cells in the epithelial cells of the thyroid at 33 to 42 h after time 0. Rats received no treatment (A) or were treated with HGF alone (B), LPS alone (C), or both HGF and LPS (D). All photographs were at a 60× original magnification. BrdU-labeled proximal tubular cells are identified with a black arrow. Red arrows identify the lumen of the tubule. (E) Quantitation of the BrdU labeling index in epithelial cells of the thyroid.

than by its age, although it remains possible that a significant effect upon crypt height might be observed at later times or if larger cohorts of animals were analyzed. The overall percentage of colonic epithelial cells that replicated in response to LPS and HGF was quite high at 48.1% of all cells. This should be sufficient to achieve efficient gene transfer if a vector that transduces dividing cells could be delivered to the colon. However, the short survival time of these cells would make it difficult to achieve a long-lasting effect. It remains unclear if the pluripotent stem cells were affected by HGF and/or LPS, and if they would be accessible to vector delivered via the blood or colonic lumen.

Thyroid

In this study, HGF alone had no effect upon replication of thyroid epithelial cells, but the combination of HGF and LPS resulted in 4.3-fold more replicating cells than were present in normal rats. The failure of normal thyroid epithelial cells to respond to HGF alone may be due to the fact that overall c-met levels are very low in the epithelial cells of the normal thyroid in most studies (53, 54), although one study did report moderate amounts of c-met in homogenates (46). In addition, the delivery of HGF to the thyroid is low after iv injection, and relatively high doses of HGF are required to observe a mitogenic response

TABLE 1

Summary of Results with HGF Alone or HGF with LPS

Organ	Expression of c-Met protein[a]	Uptake of HGF after iv injection[b]	Level of replication in HGF-treated cells relative to untreated cells[c]	Fold ↑ in LI with HGF alone[d]	Fold ↑ in LI with HGF + LPS[e]
Liver	+++	+++	Hepatocytes: 10-fold at 10 ng/ml *in vitro* (7)	17.9-fold $P = 0.001$	29.7-fold $P < 0.0001$
Lung	++	++	Alveolar cells: 2- to 7-fold at 5–20 ng/ml *in vitro* (8–10); 2- to 5-fold *in vivo* (10, 11)	2.9-fold $P = 0.0003$	9.4-fold $P = 0.0001$
			Bronchial epithelial cells: 2- to 3-fold at 30 ng/ml *in vitro* (11, 12); 3-fold *in vivo* (11)	0.7-fold NS	29-fold $P = 0.0002$
Kidney	+++	+++	Proximal tubular cells: 4-fold at 40 ng/ml *in vitro* (13, 14); 2- to 4-fold *in vivo* in acute (15–17) or chronic (18) injury models	2-fold $P = 0.007$	1.5-fold QNS
			Glomerular cells: 1.4- to 4-fold at 10–20 ng/ml *in vitro* (19, 20)	3.6-fold $P < 0.0001$	3.1-fold QNS
Colon	++ to +	+	Intestinal epithelial cells: 2-fold at 50 ng/ml (21, 22)	0.7-fold NS	2.1-fold $P = 0.001$
Thyroid	++ to ND	+	Epithelial cells: 10-fold at 50 ng/ml (23–25)	0.5-fold NS	4.3-fold $P = 0.001$
Spleen	++ to +	+++	Red pulp	0.9-fold NS	1.5-fold $P = 0.001$
			White pulp	1.6-fold NS	5.2-fold $P < 0.0001$
Thymus	ND	+	Cortex	1.3-fold NS	0.122-fold $P = 0.0005$
			Medulla	1.1-fold NS	1.2-fold NS
Pancreas	ND		Epithelial cells: 3-fold at 10 ng/ml (26, 27)	0.7-fold NS	1.1-fold NS
			Islet cells: 2-fold (28) or no change (26) *in vitro;* 2.5-fold in transgenic mice (29)	1.2-fold NS	1.0-fold NS
Skeletal muscle	ND	+	Myoblasts: 2-fold with 10 to 20 ng/ml *in vitro* (30, 31); 3-fold *in vivo* (32, 33)	1.2-fold NS	
Cardiac muscle	ND	+		1.1-fold NS	

[a] The expression of c-met protein (which is sometimes discordant with c-met mRNA) is shown based upon levels of c-met found on immunoblot (46) or HGF binding activity (47, 48) in homogenates from that organ. +++ indicates 50 to 100%, ++ represents 10 to 50%, and + represents 1 to 10% of the level in normal liver. ND represents not detected. Blank cells indicate that no data are available.

[b] Uptake of HGF after iv injection. The liver has the highest uptake of HGF after iv injection (45). The uptake in other organs at 5 min (43) or 15 min (44) after injection per gram of tissue relative to that observed in normal liver is shown using the same criteria as for c-met protein levels.

[c] The ability of primary cells, cell lines derived from that cell type, or cells *in vivo* to respond to HGF is shown. The value represents the maximal magnitude of the increase in the percentage of replicating cells or in the amount of *de novo* DNA synthesis in response to the indicated dose of HGF.

The ratio of the peak in the labeling index (LI) in response to HGF[d] or to HGF with LPS[e] to that observed in the same cell type from normal age-matched untreated rats observed in this study is shown. The *P* value for comparison of that value with normal rats is shown. NS indicates not significantly different. In a few organs, only one animal was examined from the group that received HGF with LPS. Since statistics could not be performed, these are indicated as quantity not sufficient (QNS).

to HGF in thyroid epithelial cells *in vitro*. The mechanism by which LPS potentiates the effect of HGF upon the thyroid likely involves upregulation of c-met, as LPS resulted in 4.1-fold higher levels of c-met than are present in normal thyroid. The absolute percentage of replicating thyroid epithelial cells remained moderately low at 6.7% after HGF and LPS. This would only allow a low to moderate amount of transfer of a gene into these cells with vectors that only transduce dividing cells even if the vector could have access to these cells.

Spleen

In this study, HGF had no effect upon replication in the white pulp, which consists primarily of lymphocytes, but also contains macrophages and antigen-presenting cells. However, the combination of HGF and LPS resulted in 1.5-fold as many replicating cells as in normal rats in the red pulp, which consists primarily of macrophages, endothelial cells, and lymphocytes. The need for LPS to see an effect of HGF may be due to the fact that activation of monocytes with cytokines upregulates the expression of c-met *in vitro* (55, 56), although spleen homogenates do contain large amounts of c-met. Indeed, we found that LPS resulted in 3.9-fold higher levels of c-met than are present in normal spleen. The ability of splenocytes to respond to HGF with LPS may be a reflection of the fact that the spleen has high levels of uptake of HGF after an iv injection. The relatively modest effect upon the percentage of replicating cells may be due to the fact that the two cell types in the spleen that have been reported to replicate in response to HGF, monocytes (34) and endothelial cells (35), only have a modest increase in replication at high doses. Although HGF had no effect upon replication in the white pulp, LPS resulted in 5.2-fold as many replicating cells in this region as in normal rats. This may be due to the known mitogenic effect of LPS upon B cells (42).

Induction of replication of cells in the spleen may have implications for the ability to correct some manifestations of genetic deficiencies. Indeed, we recently noted that iv injection of a retroviral vector after the IM administration of an adenoviral vector expressing HGF increased the amount of retroviral vector DNA and RNA in the spleen over that observed in normal mice that received the same dose of retroviral vector (57). Administration of early generation adenoviral vectors induces many aspects of the acute phase response (58–60), and thus probably resembles the effect of LPS. Augmentation of splenocyte transduction could be important for correcting lysosomal storage diseases, where the spleen is a major site of pathology. Transduction of splenocytes could also be important for inducing cytotoxic T lymphocyte (CTL) responses, as they are reported to require transduction of an antigen-presenting cell (61, 62). This could have adverse consequences for gene therapy for inherited genetic deficiencies, or positive effects in gene therapy for cancer. As it will be critical to identify the specific cell types whose replication is stimulated by HGF and/or LPS *in vivo*, studies to define the phenotype of these cells is currently being investigated using immunocytochemistry.

Thymus

In this study, HGF failed to induce replication of thymocytes, while LPS resulted in a marked reduction in the percentage of replicating cells in the cortex. The combination of HGF and LPS had a similar effect to that of LPS alone. Although HGF promotes maturation of fetal thymocytes *in vitro* (63), its effect upon replication of adult thymocytes has not been reported. The fact that c-met receptor RNA levels are low in the adult thymus of mice (63) suggests that these cells may not express the receptor, which could be responsible for the absence of an effect here. The LPS-induced reduction in the percentage of replicating cells in the thymus may have been due to the induction of apoptosis. Some studies have injected adenoviral vectors into the thymus in order to induce tolerance to the transgene (64). Since a significant fraction of thymocytes are replicating with or without HGF, it might be possible to use MLV retroviral vectors to transfer genes into the thymus for induction of tolerance.

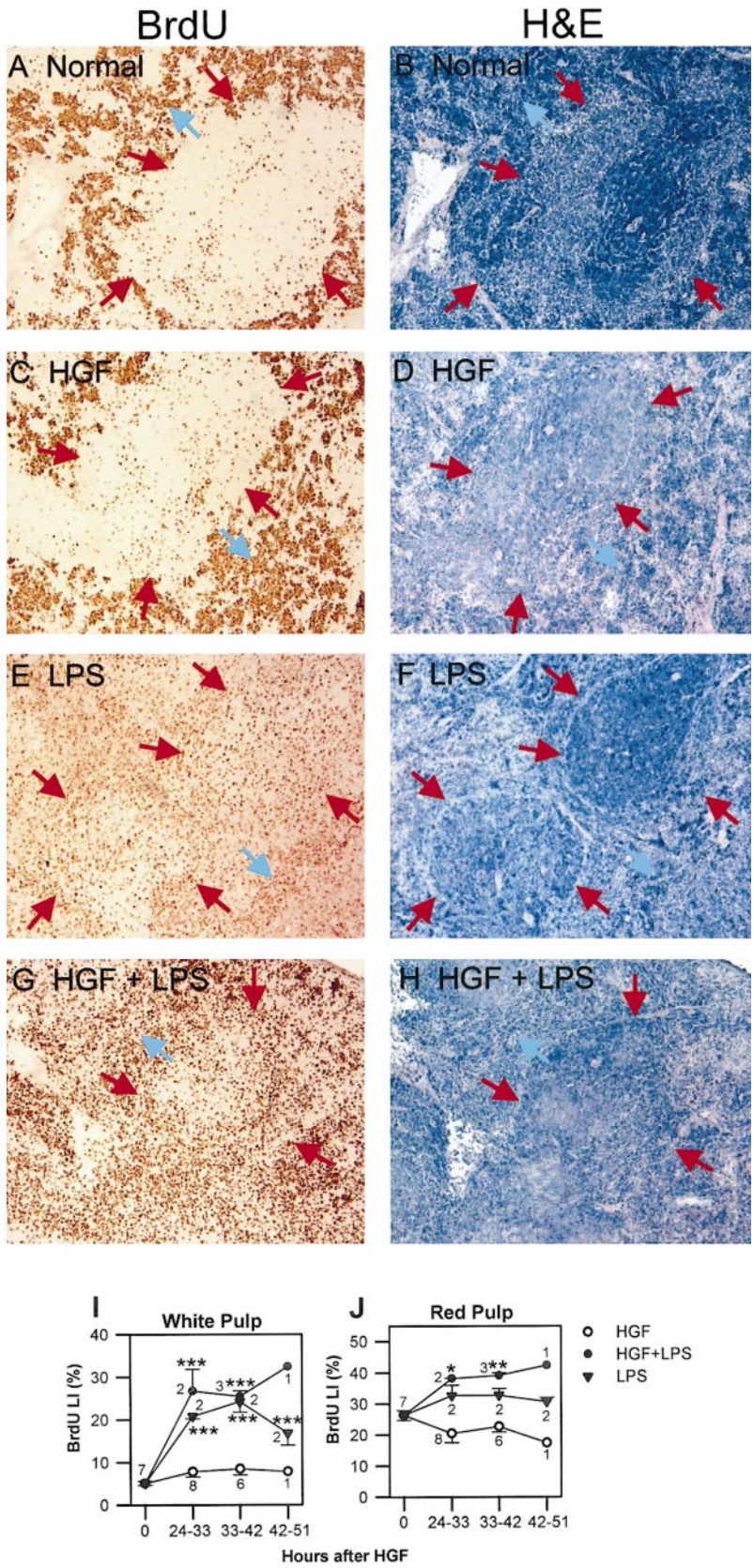
Muscle, Pancreas, and Blood Vessels Did Not Respond to iv HGF

Myoblasts, pancreatic epithelial cells, and pancreatic islet cells have been reported to replicate in response to HGF *in vitro*. However, HGF alone had no effect upon replication in skeletal or cardiac muscle, or in epithelial or endocrine cells of the pancreas in this study (Table 1). This poor response may be due to the fact that both organs have undetectable levels of uptake of HGF after iv injection, and the muscle is known to have low levels of c-met protein. Although c-met protein levels relative to other organs have not been reported, the adult pancreas has low levels of c-met RNA (65), and thus likely has low protein levels. Although inflammation can upregulate expression of c-met in the pancreas (66), LPS did not enable HGF to induce replication in the pancreas *in vivo* in this study.

Endothelial cells have been reported to replicate in response to HGF (35), and are of particular importance as they would have direct contact with a retroviral vector that might be administered iv for hepatic gene therapy. However, we failed to observe labeled cells on the surface or within the wall of blood vessels (data not shown). This is likely due to the fact that endothelial cells only respond to very high doses of HGF [100 ng/ml (35)], and the levels of HGF that were achieved were insufficient to have an effect.

Implications for Gene Therapy

This study has two major implications for using HGF for gene therapy. First, HGF alone should be optimal for facilitating gene transfer into the liver. HGF alone was almost as effective as the combination of HGF and LPS upon inducing replication in the liver (5). In addition, HGF alone was almost as efficient at facilitating transduction with a retroviral vector as was the combination of HGF and LPS. HGF alone resulted in transduction of $0.63 \pm 0.2\%$ of hepatocytes after iv injection of a cumulative dose of 7×10^8 infectious particles of a β-galactosidase-expressing vector as 5 doses between 27 and 39 h after the first dose of HGF into young adult rats (data not shown), which was 18.3-fold higher than the transduc-



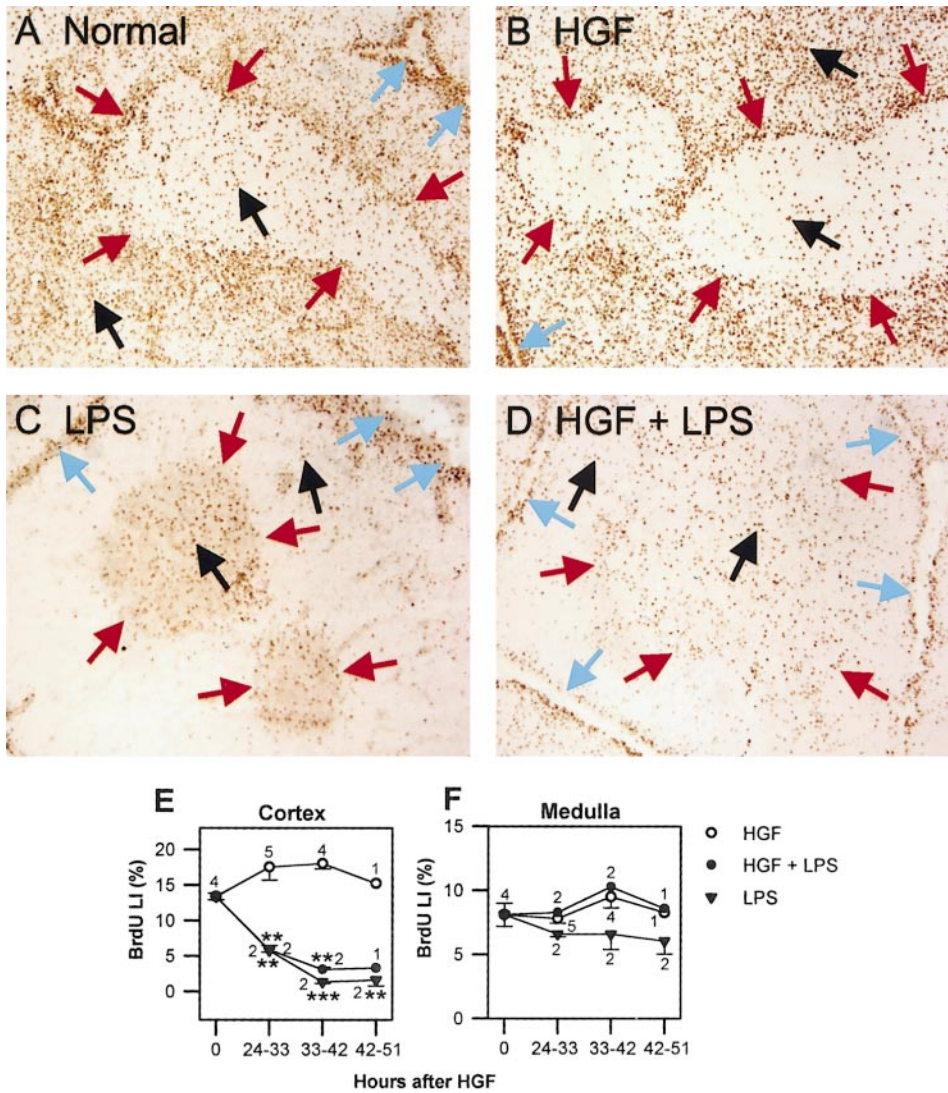


FIG. 8. (A–D) Examples of replication in the thymus. Rats were treated as noted in the legend to Fig. 2, and anti-BrdU followed by eosin staining was performed to determine the percentage of replicating cells in the thymus at 33 to 42 h after time 0. BrdU-labeled cells appear as small brown dots that are identified with a black arrow and are more prevalent in the cortex of normal rats. The outer edges of the medulla, which is located in the center of each panel, are identified with red arrows. The edge of the lobule is identified with blue arrows. Rats received no treatment (A) or were treated with HGF alone (B), LPS alone (C), or both HGF and LPS (D). All photographs were at a 10× original magnification. (E) Quantitation of the BrdU labeling index in cortex of the thymus. (F) Quantitation of the BrdU labeling index in the medulla of the thymus.

tion that was observed in rats that did not receive HGF prior to retroviral vector (data not shown). The combination of HGF and LPS resulted in transduction of 0.72 ± 0.2% of hepatocytes with the same regimen of retroviral vector, which was only 14% higher than what was observed with HGF alone. In addition, HGF alone had a much more potent effect upon replication in hepatocytes than in other cells. The absent or modest effect upon replication in other cells suggests that HGF alone should not adversely affect the structure or function of other organs, although it remains possible that HGF could exert a morphogenic or motogenic effect in cells that do not

FIG. 7. (A, C, E, and G) Examples of replication of cells in the spleen. Rats were treated as noted in the legend to Fig. 2. Anti-BrdU immunostaining followed by a light eosin counterstain (A, C, E, and G) was performed to determine the percentage of replicating cells in the spleen at 33 to 42 h after time 0. The BrdU-labeled cells appear as small brown dots. An adjacent section was stained with H & E to help to identify the different regions of the spleen. The red arrows indicate the outer edge of the white pulp, which is located in the center of all panels. The blue arrow identifies a region of red pulp. Rats received no treatment (A and B) or were treated with HGF alone (C and D), LPS alone (E and F), or both HGF and LPS (G and H). All photographs were at a 10× original magnification. (I) Quantitation of the BrdU labeling index in the white pulp. (J) Quantitation of the BrdU labeling index in the red pulp.

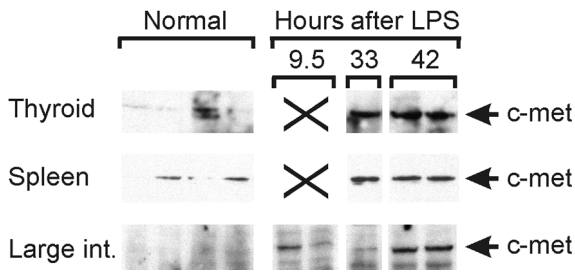


FIG. 9. The effect of LPS upon c-met protein levels. Extracts from thyroid, spleen, or large intestine (Large int.) were harvested from normal rats or at the indicated times after treatment with 5 mg/kg of LPS. Immunoblot was performed on 50 to 100 μg of protein after electrophoresis on a reducing SDS–PAGE using an anti-c-met antibody. The position of the 145-kDa c-met protein is indicated at the right.

exhibit a robust mitogenic response to HGF. Finally, the use of HGF alone did not involve the administration of a toxic reagent.

The second implication of this study is that iv HGF alone will not be sufficient to facilitate efficient transduction in other organs with vectors that only transduce dividing cells, as other cells had either a modest or no response to HGF alone. In contrast, the combination of HGF and LPS might be effective, as it markedly increased replication in some cell types. This potentiation by LPS may involve upregulation of c-met, as several cells have low expression of c-met in the basal state, but are induced to express it by inflammation or cytokines (50, 51, 55, 56, 66). Indeed, we found that LPS increase levels of c-met in the spleen, thyroid, and large intestines. Alternatively, the administration of LPS and HGF may result in synergistic activation of signal transduction pathways, as we hypothesized occurs in the liver (5). The requirement for two signals to achieve replication may be a mechanism to ensure that replication does not occur inappropriately when HGF alone is increased. Although this dose of LPS caused little overt toxicity in rats, it resulted in a transient decrease in levels of some liver transcription factors and expression of some liver-specific genes (B. Wang, C. Gao, and K. P. Ponder, unpublished data), and would likely be inappropriate for use in human gene therapy. It will therefore be necessary to try to identify downstream mediators of the acute phase response such as IFNγ that might have a similar effect with less toxicity. In addition, it may be necessary to delivery the vector locally, as iv injection would likely not allow the vector to reach the parenchymal cells in most organs.

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