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*Kidney International, Vol. 53 (1998), pp. 350–357*

# Morphine modulates proliferation of kidney fibroblasts

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**Morphine modulates proliferation of kidney fibroblasts.** Renal interstitial scarring is an important component of heroin-associated nephropathy. Kidney fibroblasts have been demonstrated to play a role in the development of renal scarring in a variety of renal diseases. We studied the effect of morphine, an active metabolite of heroin, on the proliferation of kidney fibroblasts. Morphine at a concentration of $10^{-12}$ M enhanced ($P < 0.001$) the proliferation of kidney fibroblasts (control, 67.5 ± 2.0 vs. morphine, 112.2 ± 10.1 × $10^4$ cells/well). [$^3$H]thymidine incorporation studies further confirmed these results. Morphine at concentrations of $10^{-12}$ M to $10^{-10}$ M also modulated mRNA expression of early growth related genes (c-*fos*, c-*jun* and c-*myc*). Morphine at concentrations of $10^{-8}$ to $10^{-4}$ M promoted apoptosis of kidney fibroblasts and also enhanced the synthesis of p53 by kidney fibroblasts. We speculate that morphine-induced kidney fibroblast proliferation may be mediated through the activation of early growth related genes, whereas morphine induced kidney fibroblast apoptosis may be mediated through the generation of p53. The present *in vitro* study provides a hypothetical basis for the role of morphine in the development of renal interstitial scarring in patients with heroin-associated nephropathy.

Heroin-associated focal glomerulosclerosis is accompanied by interstitial fibrosis [1, 2]. Renal interstitial fibrosis has also been demonstrated to be a better marker of renal disease progression than glomerular scarring in a variety of renal diseases [3, 4]. Recently, the mechanism of heroin associated glomerular lesions has been investigated in depth [5–10]. However, the role of heroin in the induction of interstitial fibrosis has not been investigated [5].

Kunico et al suggest that normal wound healing serves as a model for the development of tissue fibrosis [11]. Muller et al also emphasize that the same process may be operating in the development of renal fibrogenesis [12]. The wound healing process consists of three phases: induction of tissue injury, inflammation with deposition of extracellular matrix, and resolution of the inflammatory process. In the case of renal injury there may be an immunologic mechanism involving glomeruli, tubulointerstitium, or vasculature resulting in an influx of mononuclear cells, predominantly CD4+ T-lymphocytes (induction phase). Infiltration of these activated cells causes a local release of chemokines, which not only perpetuates the accumulation of these cells but also invites fibroblasts to become a major player in the process. In addition, the release of cytokines activates resident fibroblasts [13]. Activated fibroblasts proliferate and synthesize matrix components [12, 14]. However, with the termination of the primary insult, this process may be reversible due to increased matrix degradation and decreased release of cytokines during the resolution phase as has been demonstrated in the animal model of puromycin aminonucleoside-induced nephrosis [15]. However, if the primary injury persists or the release of cytokines continues, so will the accumulation of matrix eventually leading to the accumulation of altered extracellular matrix. The latter may change the function of cells and thus may hamper the recovery process.

Important mitogens for fibroblasts include insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF), endothelin-1, epidermal growth factor (EGF), and transforming growth factor-$\alpha$ (TGF-$\alpha$) [16–18]. Fibroblasts in primary cultures derived from kidneys with early stages of interstitial renal disease show altered proliferation of fibroblasts compared to fibroblasts derived from normal kidneys [19].

Morphine is an active metabolite of heroin and has been incriminated for the effect of heroin on target organs [20]. In the past, we examined the effect of heroin as well as morphine in animal experiments [5]. Since the majority of cells in culture are unable to metabolize heroin to morphine, we preferred to use morphine in *in vitro* experiments. Data on the effect of morphine on fibroblasts in general is scanty and on kidney fibroblasts, in particular, is not available [21]. In the present study we examined the effect of morphine on the proliferation as well as apoptosis of kidney fibroblasts.

## METHODS

### Kidney fibroblasts

Cultured rat kidney fibroblasts (NRK-49F ATCC Cat # CRL 1570) were obtained from the American Tissue Cell Collection (Rockville, MD, USA). These cells were plated in 75 cm$^2$ flasks and incubated in media containing DMEM (GIBCO, Grand Island, NY, USA), penicillin (50 units/ml; GIBCO), streptomycin (50 $\mu$g/ml; GIBCO) and 10% fetal calf serum (GIBCO) in an environment of 5% $CO_2$ and 95% air at 37°C. Kidney fibroblasts were allowed to grow to confluence for five to seven days before being subcultured.

### Source of reagents

Morphine was dissolved in normal saline, stocked at a concentration of $10^{-2}$ M and used in concentrations of $10^{-14}$ to $10^{-4}$ M

**Key words:** morphine, fibroblasts, heroin-associated nephropathy, renal scarring HIV-associated nephropathy.

Received for publication April 30, 1997
and in revised form September 9, 1997
Accepted for publication September 10, 1997

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(National Institute on Drug Abuse, Bethesda, MD, USA); [$^3$H]thymidine was stocked in a concentration of 16.3 Ci/mmol (Dupont NEN, Cambridge, MA, USA); Hoechst (H)-33342 was used in a concentration of 1 $\mu$g/ml (Molecular Probes, Inc., Eugene, OR, USA); propidium iodide was used in a concentration of 1 $\mu$g/ml (Sigma Chemical Company, St. Louis, MO, USA); mouse anti-p53 antibody was used in a concentration of 1 $\mu$g/ml (Oncogene Research Products, Cambridge, MA, USA). The APOBrDU kit was obtained from Pharmingen (San Diego, CA, USA).

### Cell proliferation studies

Equal numbers of kidney fibroblasts (10,000 cells/well) were plated in 24-well culture plates and incubated in Dulbecco's modified Eagle medium containing 10% fetal calf serum (FCS), penicillin and streptomycin. Cells grew to subconfluence within two to three days. Subconfluent cells were growth arrested for 72 hours by incubating in DMEM containing 1% ITS (insulin, selenium, transferrin) and 0.5% bovine serum albumin (BSA). Cells were washed twice with phosphate buffered saline (PBS) and incubated in RPMI 1640 containing either buffer alone (control) or morphine ($10^{-12}$ M to $10^{-4}$ M) for 24 hours at 37°C. At the end of the incubation period, the cells were washed twice with PBS, trypsinized and counted in a hemocytometer. Six sets of experiments (each in quadruplicate) were carried out.

To determine the effect of morphine on mitotic index of kidney fibroblasts, equal numbers of growth arrested subconfluent cells were incubated with media containing either buffer alone (control), or variable concentrations of morphine ($10^{-14}$ to $10^{-6}$ M) for 24 hours. At the end of the incubation period, cells having two nuclei were counted in eight random fields. We calculated the percentage of cells having two nuclei in each condition. Four sets of experiments were carried out, each in triplicate.

To determine the effect of morphine on the size of kidney fibroblasts we evaluated the number of cells per microgram of cellular protein with or without morphine treatment. Subsequently, we calculated the mean protein content of each cell in different groups. In brief, equal numbers of growth arrested subconfluent cells were incubated in media containing either buffer or variable concentrations of morphine ($10^{-14}$ to $10^{-6}$ M) for 48 hours. At the end of the incubation period, cells were trypsinized and an aliquot from each sample was counted in a hemocytometer and the total number of cells in each well was determined. The rest of the cells were washed twice with PBS and pelleted after centrifugation. Protein content of each pellet was assayed by a BCA kit (Pierce) and the number of cells per microgram of cellular protein was calculated for each sample. Four sets of experiments were performed, each in triplicate.

### [$^3$H]thymidine incorporation studies

To determine the dose response effect of morphine on the synthesis of DNA by kidney fibroblasts, equal numbers of cells (10,000 cells/well) were plated on 24 well plates. Cells were grown to subconfluence and then growth arrested for 72 hours. Cells were washed twice with PBS and incubated in DMEM containing either buffer alone (control) or variable concentrations of morphine ($10^{-14}$ to $10^{-6}$ M) for 48 hours at 37°C. At 42 hours, an aliquot of DMEM containing 0.25 $\mu$Ci of [$^3$H]thymidine was added to each well and incubation continued for six hours. At the end of the incubation period cells were washed three times with cold PBS, 200 $\mu$l of 5% trichloroacetic acid (TCA) added to each well and cells incubated at 4°C for 60 minutes. The TCA supernatant was removed and 200 $\mu$l of 0.25 N NaOH was added to each well. The cells were kept at room temperature overnight after which the contents of each well was counted in a scintillation counter. Results of thymidine incorporation were used as a measure of cellular proliferation. Three sets of experiments (four wells for each experiment) were carried out.

### H-33342 and propidium iodide staining

H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence, whereas propidium iodide co-stains dead cells. Double staining by these two agents provides the percentage of live, apoptotic and necrosed cells under control and experimental conditions. To determine the effect of morphine on kidney fibroblast apoptosis, equal numbers of cells (10,000 cells/well) were plated in 24-well plates. After 48 hours, cells were washed twice with PBS and incubated in media (DMEM + 1% FCS) containing either buffer (control) or variable concentrations of morphine ($10^{-10}$ M to $10^{-4}$ M) for 24 hours. At the end of the incubation period, cells were incubated with H-33342 (1 $\mu$g/ml) for seven minutes at 37°C. Subsequently cells (without a wash) were placed on ice and propidium iodide (final concentration, 1 $\mu$g/ml) was added to each well. Cells were incubated with the dyes for 10 minutes on ice, protected from light, and examined under ultraviolet light. The percentage of live, apoptotic and necrosed cells were recorded in eight random fields. Nine hundred to 1400 cells were examined for different variables in each experiment. Four sets of experiments were carried out.

### Detection of kidney fibroblast apoptosis by the TUNEL method

Using the TUNEL (Terminal deoxy transferase Uridine triphosphate Nick End-labeling) method, subconfluent kidney fibroblasts were incubated in media (DMEM + 1% FCS) containing either buffer (control) or variable concentrations of morphine ($10^{-10}$ to $10^{-6}$ M) for 24 hours. At the end of the incubation period, cells were stained using an APOBrDU kit (Pharmingen, San Diego, CA, USA). Percentage of apoptotic cells was recorded under an immunofluorescence microscope. Four sets of experiments were performed.

### Detection of fibroblast apoptosis by DNA end-labeling

This is a sensitive method for detection of apoptosis. Briefly, equal numbers of fibroblasts were plated on 100 mm Petri dishes containing media for 24 hours. Subsequently, the cells were washed twice with PBS and incubated in media containing either buffer (control) or morphine ($10^{-12}$ M to $10^{-6}$ M) for 24 hours. At the end of the incubation period, the cells were washed and centrifuged at 800 rpm for 10 minutes at room temperature. The supernatants were discarded, the pellet resuspended in DNA lysis buffer [10 mM Tris, pH 7.4, 1.5 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0, and 1% SDS) and incubated with 5 $\mu$l/ml proteinase K (Promega, Madison, WI, USA] overnight at 37°C. The DNA was extracted twice with phenol:chloroform (1:1) and twice with chloroform:isoamyl alcohol (24:1). DNA was precipitated overnight at −20°C in a 0.3 mM final concentration of Na acetate, 8 $\mu$l of 1 M $MgSO_4 \cdot 7H_2O$ and 2.5 volumes of absolute ethanol. The samples were centrifuged at 10,000 rpm for 30 minutes at 4°C, in an Eppendorf 5415C centrifuge and washed twice with 70% ethanol. The pellets were dried in a speed-vac for

10 minutes. The pellets were resuspended in TE buffer (10 mM Tris and 1 mM EDTA) and DNA concentrations were determined by reading the absorbance at 260 nm. Five micrograms of isolated DNA from control and morphine treated fibroblasts were end-labeled with 5U Klenow polymerase (Promega) in the presence of 10 mM Tris (pH 7.5), 5 mM $MgSO_4 \cdot 7H_2O$, and 0.5 $\mu$l of $^{32}$P dCTP (DuPont NEN, Boston, MA, USA) for 10 minutes at room temperature. The reaction was stopped by adding 10 mM EDTA. The labeled DNA were purified by Quick Spin nucleotide removal columns (Qiagen). The end-labeled DNA were electrophoresed on a 1.8% agarose gel at 5 volts per cm in 0.5X TE buffer containing 10 $\mu$g/ml ethidium bromide. After drying the gel on a slab gel dryer for 45 minutes at 40°C, radiolabeled fragments were visualized by exposure to Kodak X-ray film at −70°C for 30 minutes to three hours.

### Protein extraction

Equal numbers (10,000) of kidney fibroblasts were seeded in 100 mm Petri dishes and grown to subconfluence. Subsequently, cells were washed twice with PBS and incubated in medium containing either buffer (control) or morphine ($10^{-12}$ M to $10^{-4}$ M) for two hours. In parallel experiments, equal numbers of subconfluent kidney fibroblasts were incubated with morphine ($10^{-12}$ M and $10^{-8}$ M) for variable time periods (2, 4 and 6 hr). At the end of the incubation periods, cells were washed twice with PBS and incubated with 100 $\mu$l of PBSTDS lysis buffer for 30 minutes at room temperature. Cells were scraped and protein was assayed with a BCA kit (Pierce, Rockford, IL, USA).

### Western blotting

The proteins (20 $\mu$g/lane) extracted from cell lysates were separated on 4 to 20% gradient polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane using a BIORAD Western blotting apparatus. Nitrocellulose membranes were then processed further for p53 using mouse anti-p53 at 1 $\mu$g/ml concentration, one hour at room temperature using HRP (horseradish peroxidase) labeled with secondary goat anti-mouse antibody (Pierce), and then the blots were developed using chemiluminescence (ECL; Amersham).

### RNA extraction and identification of growth associated genes

Equal numbers of subconfluent and growth-arrested cultured rat kidney fibroblasts were treated either with vehicle (control) or morphine ($10^{-12}$ M and $10^{-10}$ M) for 10, 30, 60 and 120 minutes. At the end of the incubation periods, cells were washed twice with PBS and then treated with lysis buffer. Total RNA was extracted from lysates of cultured rat kidney fibroblasts. Aliquots of total RNA were treated with formamide and formaldehyde, electrophoresed in a 1.2% agarose-formaldehyde gel, and transferred to HYBOND-N membranes. The gel was stained with ethidium bromide to determine the size of RNA. cDNA probes specific for c-*fos*, c-*jun* and c-*myc* (gift from Dr. Sanjeev Gupta, Liver Research Center, Albert Einstein College of Medicine, Bronx, NY, USA) were used for hybridization after [$^{32}$P]dCTP labeling by random-prime labeling. Filters were hybridized at 42°C for 16 hours with the labeled specific cDNA probe. The membranes were washed to a final high stringency of 0.2 × SSC, 0.1% SDS for 20 minutes at 65°C. After washing, the membranes were kept in contact with XAR-5 film and intensifying screen at −70°C and developed. The membranes were stripped to remove the hybridized probe and reprobed with GAPDH to ascertain that similar amounts of RNA were applied to the gel. Densitometric analysis of the ratios between c-*fos*/c-*jun*/c-*myc* and GAPDH expression was determined for control as well as morphine treated cells. Three sets of experiments were carried out and results were calculated as mean ± SEM.

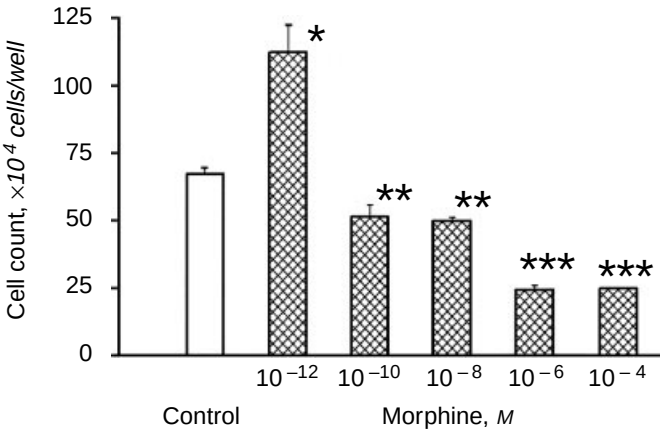


**Fig. 1. Effect of morphine on kidney fibroblast proliferation at 24 hours.** Equal numbers of growth arrested kidney fibroblasts were incubated in DMEM containing either buffer alone (control) or variable concentrations of morphine ($10^{-12}$ M to $10^{-4}$ M) for 24 hours at 37°C ($N = 6$). *$P <$ 0.001 compared with control and morphine $10^{-10}$ M to $10^{-4}$ M; **$P < 0.01$ compared with morphine, $10^{-6}$ M to $10^{-4}$ M; ***$P < 0.001$ compared with control.

### Statistical analysis

Comparison of cultured rat kidney fibroblast proliferation and matrix accumulation between control and experimental conditions was carried out by an unpaired Student's *t*-test. When more than two groups were involved, intergroup comparisons were performed by analysis of variance. A Newman-Keuls multiple range test was used to calculate a q value. Results are represented as means ± SEM. Difference was considered to be statistically significant at $P < 0.05$.

## RESULTS

### Effect of morphine on proliferation of kidney fibroblasts

The 24 hour effect of morphine on the proliferation of fibroblasts is shown in Figure 1. Morphine at higher concentrations ($10^{-6}$ M to $10^{-4}$ M) induced suppression of kidney fibroblast proliferation (Fig. 1). Morphine enhanced ($P < 0.001$) the proliferation of kidney fibroblasts at a lower concentration (morphine, $10^{-12}$ M, 112.2 ± 10.1 vs. control, 67.5 ± 2.0 × $10^4$ cells/well; 24 hr). Similarly, at 48 hours, morphine enhanced kidney fibroblast proliferation at lower concentrations (Fig. 2). This was further evident from the 48 hour effect of morphine on the synthesis of DNA (Fig. 3). At lower concentrations, morphine treated cells showed a greater percentage of mitotic index when compared to untreated cells (Fig. 4). However, at lower concentrations morphine treated cells contained a much lower mean protein content when compared to control cells (control, 0.66 ± 0.15; morphine $10^{-14}$ M, 0.44 ± 0.01; morphine, $10^{-12}$ M, 0.23 ± 0.02; morphine, $10^{-10}$ M, 0.39 ± 0.02; morphine, $10^{-8}$ M, 0.47 ± 0.11; morphine, $10^{-6}$ M, 0.88 ± 0.08 ng protein/cell). A decrease in the

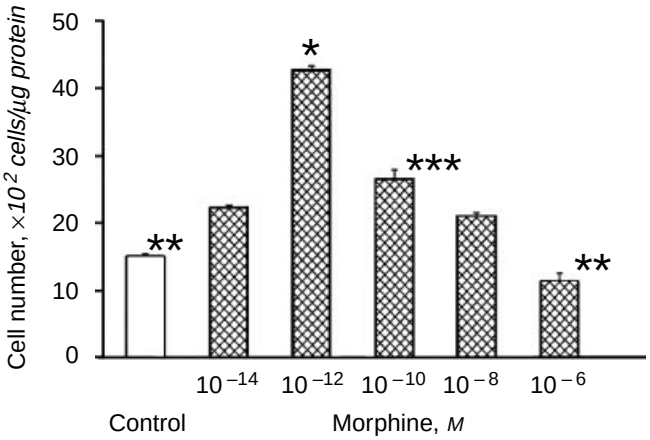


**Fig. 2. Effect of morphine on kidney fibroblast proliferation at 48 hours.** Equal numbers of growth arrested subconfluent kidney fibroblasts were incubated in media containing either buffer (control) or variable concentrations of morphine ($10^{-14}$ to $10^{-6}$ M) for 48 hours. At the end of the incubation period, number of cells as well as protein content per well were measured ($N = 4$). $^{**}P < 0.001$ compared with morphine, $10^{-14}$ to $10^{-8}$ M; $^{*}P < 0.001$ compared with morphine, $10^{-14}$ M, $10^{-10}$ M and $10^{-8}$ M; $^{***}P < 0.05$ compared with morphine, $10^{-14}$ M and $10^{-8}$ M.

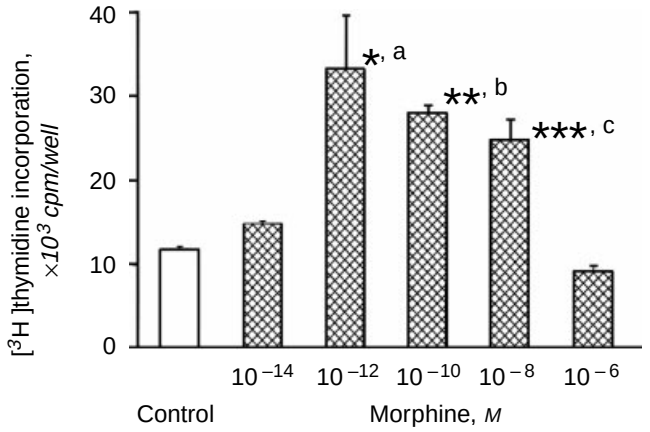


**Fig. 3. Effect of morphine on kidney fibroblast DNA synthesis.** Equal numbers of growth arrested kidney fibroblasts were incubated in media (DMEM) containing either buffer alone (control) or variable concentrations of morphine ($10^{-14}$ to $10^{-6}$ M) for 48 hours at 37°C. At 42 hours, an aliquot of media containing [$^3$H]thymidine was added to each variable. At the end of the incubation period cells were harvested and radioactivity was measured ($N = 3$). $^{*}P < 0.001$ compared with control and morphine, $10^{-6}$ M; $^{**}P < 0.01$ compared with control and $10^{-6}$ M. $^{***}P < 0.05$ compared with control; $^{a}P < 0.01$ compared with morphine, $10^{-14}$ M; $^{b}P < 0.05$ compared with morphine, $10^{-14}$ M; $^{c}P < 0.01$ compared with morphine, $10^{-6}$ M.

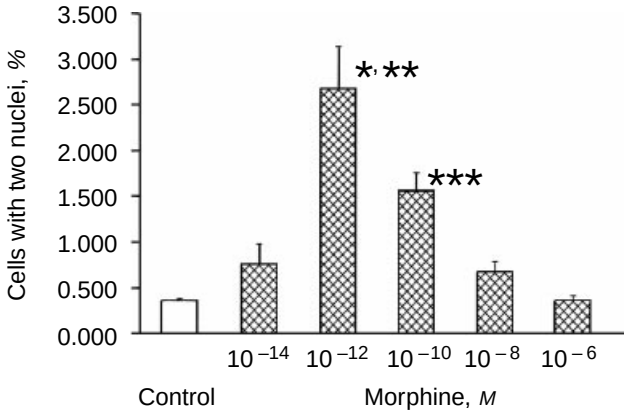


**Fig. 4. Effect of morphine on kidney fibroblast mitotic index.** Equal numbers of growth arrested kidney fibroblasts were incubated in media containing either buffer (control) or variable concentrations of morphine ($10^{-14}$ to $10^{-6}$ M) for 24 hours. At the end of the incubation period, the percentage of cells with two nuclei was counted ($N = 4$). $^{*}P < 0.001$ compared with control, morphine, $10^{-14}$ M, $10^{-8}$ to $10^{-6}$ M; $^{**}P < 0.01$ compared with morphine, $10^{-10}$ M; $^{***}P < 0.05$ compared with control, morphine, $10^{-14}$ M, $10^{-8}$ to $10^{-6}$ M.

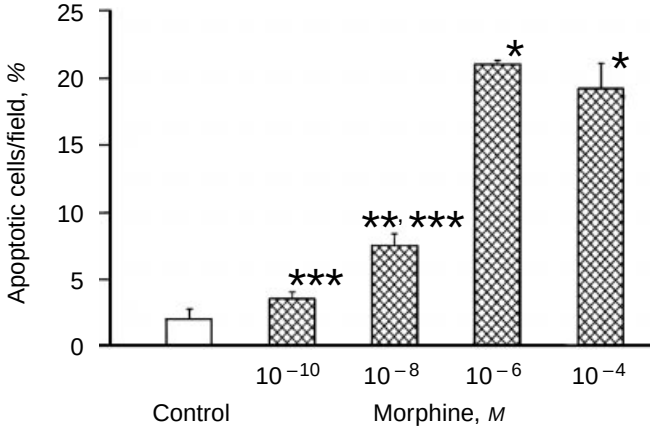


**Fig. 5. Effect of morphine on fibroblast apoptosis.** Equal numbers of kidney fibroblasts were incubated in media (DMEM + 1% FCS) containing either buffer (control) or variable concentrations of morphine ($10^{-10}$ M to $10^{-4}$ M) for 24 hours. At the end of the incubation period, cells were stained with H-33342 (1 μg/ml) and propidium iodide. Percentage of live, apoptotic and necrosed cells were recorded in eight random fields ($N = 4$). $^{*}P < 0.001$ compared with control; $^{**}P < 0.05$ compared with control; $^{***}P < 0.001$ compared with morphine, $10^{-6}$ M and $10^{-4}$ M.

mean cell protein content in the morphine ($10^{-14}$ to $10^{-12}$ M) treated group may be related to the accumulation of an increased number of younger (dividing) cells with a smaller size.

### Effect of morphine on kidney fibroblast apoptosis using the TUNEL (APOBrdu) method

Morphine in concentrations of $10^{-8}$ to $10^{-6}$ M showed enhanced apoptosis of kidney fibroblasts at 24 hours (control, 0.8 ± 0.1%, morphine, $10^{-10}$ M, 2.1 ± 0.7%; morphine, $10^{-8}$ M, 5.6 ± 1.0%; morphine, $10^{-6}$ M, 9.0 ± 0.7%; $P < 0.001$, control vs. morphine, $10^{-6}$ M; $P < 0.01$, control vs. morphine, $10^{-8}$ M). These results further confirm that morphine at higher concentrations promotes kidney fibroblast apoptosis.

### Effect of morphine on apoptosis of kidney fibroblasts by H-33342 staining

The effect of morphine on kidney fibroblast apoptosis at 48 hours is shown in Figure 5. Morphine promoted apoptosis of kidney fibroblasts at higher concentrations ($10^{-8}$ M to $10^{-4}$ M). The percentage of apoptotic cells was higher at 48 hours when compared to the percentage of apoptotic cells at 24 hours (TUNEL method). These results suggest that morphine-induced

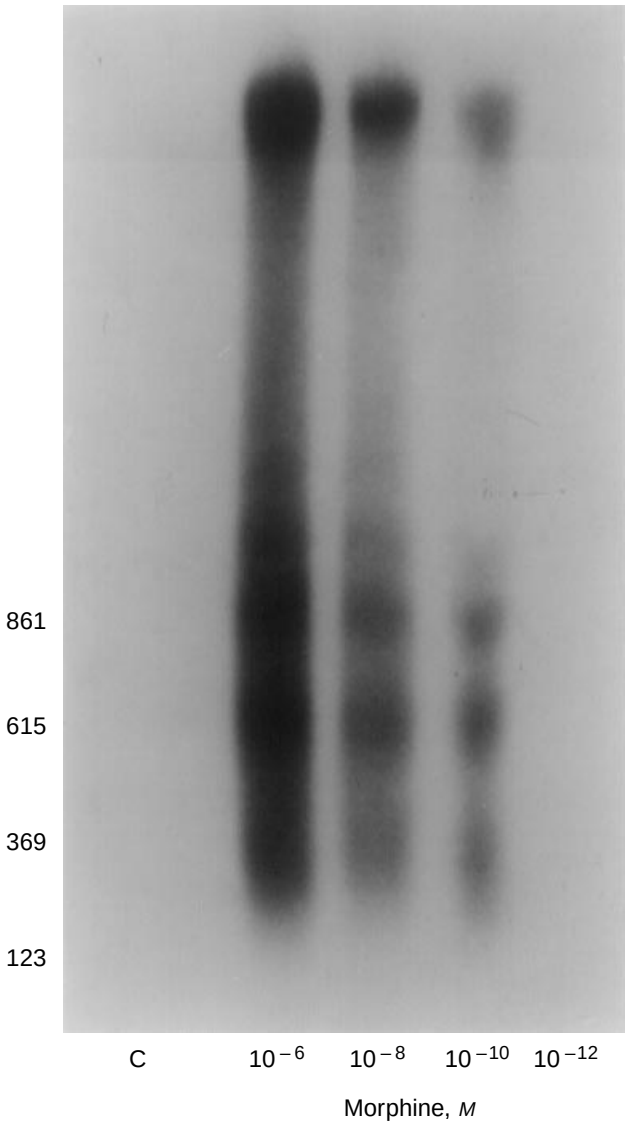


**Fig. 6. DNA end-labeling of control and morphine treated kidney fibroblasts.** DNA was isolated from control (C) and morphine ($10^{-12}$ to $10^{-6}$ M) treated kidney fibroblasts and end-labeled. Morphine ($10^{-10}$ M to $10^{-6}$ M) treated fibroblasts showed a classic ladder pattern (integer multiples of 180 base pairs).

apoptosis of kidney fibroblasts may be contributing to morphine-induced suppression of fibroblast proliferation.

### Effect of morphine on fibroblast DNA fragmentation

The effect of morphine on fibroblast DNA fragmentation (DNA end-labeling) is shown in Figure 6. Control cells (Lane C) did not show any DNA fragmentation. Fibroblasts treated with higher concentrations ($10^{-10}$ M to $10^{-6}$ M) of morphine showed DNA fragmentation (integer multiples of 180 base pairs) in the form of a ladder pattern.

### Effect of morphine on the synthesis of p53 by kidney fibroblasts

The effect of morphine on the synthesis of p53 by kidney fibroblasts is shown in Figures 7 and 8. Morphine enhanced the

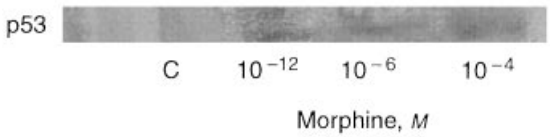


**Fig. 7. Effect of morphine on synthesis of p53 by kidney fibroblasts (Western blot).** Equal numbers of kidney fibroblasts were seeded in 100 mm Petri dishes and grown to subconfluence. Equal numbers of subconfluent and growth arrested cells were incubated in medium containing either buffer (control), or morphine ($10^{-12}$ M to $10^{-4}$ M) for two hours. At the end of the incubation, cells were lysed, proteins were extracted and probed using mouse anti-p3 (1 $\mu$g/ml). Western blot shows that morphine at higher concentrations enhances the production of p53.

synthesis of p53 by kidney fibroblasts in a dose dependent manner (Fig. 7). Morphine treated cells at a lower concentration ($10^{-12}$ M) showed minimal synthesis of p53 by kidney fibroblasts, while morphine at a concentration of $10^{-4}$ M induced the maximal synthesis of p53 by cultured rat kidney fibroblast cells. Synthesis of p53 by morphine treated kidney fibroblasts showed a plateau at four hours (Fig. 8). Since morphine promoted apoptosis only at higher concentrations, it appears that morphine-induced enhanced synthesis of p53 may be playing a role in the induction of apoptosis.

### Effect of morphine on mRNA expression of early growth related genes

The effects of morphine on mRNA expression of early growth related genes are shown in Figures 9, 10, 11 and Table 1 (means ± SEM are from three sets of experiments). Morphine ($10^{-10}$ M) increased mRNA expression of c-*jun* by 1.7-fold at 10 minutes (Fig. 9 and Table 1), whereas morphine ($10^{-12}$ M) enhanced expression of c-*fos* by 2.3-fold (Fig. 10 and Table 1). Morphine ($10^{-10}$ M) increased expression of c-*myc* by 1.6-fold at 120 minutes (Fig. 11 and Table 1).

## DISCUSSION

The present study demonstrates that morphine modulates kidney fibroblast proliferation. Morphine enhances proliferation at lower concentrations and causes apoptosis at higher concentrations. Morphine at lower concentrations also modulates mRNA expression of c-*fos*, c-*jun* and c-*myc* on kidney fibroblasts. Morphine at higher concentrations enhances the production of p53 by kidney fibroblasts.

Morphine has been reported to induce a direct modulatory effect on the proliferation of different cells of immune origin [22–24]. Recently, DiFrancesco et al demonstrated that morphine blocks the entry of stimulated EL2 rat fibroblasts from G0/G1 to S1 phase [21]. However, these investigators did not study the effect of morphine on fibroblasts under a basal state. Moreover, these investigators used morphine at high concentrations which are likely to cause suppression of fibroblast proliferation. Morphine at pharmacological doses provides a blood concentration of $10^{-8}$ M or lower. The effect of these concentrations was not evaluated by these investigators. On the contrary, we have used morphine at pharmacological ($10^{-8}$ M) as well as at lower concentrations (physiological, $10^{-12}$ M) in the present study. At higher concentrations morphine suppresses the proliferation of fibroblasts and our results are consistent with DiFrancesco et al [21]. Since morphine promotes apoptosis of fibroblasts at higher

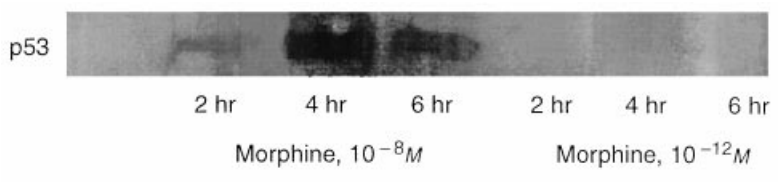


**Fig. 8. Time course effect of morphine on the production of p53 by kidney fibroblasts (Western blot).** Equal numbers of subconfluent and growth arrested kidney fibroblasts were incubated with morphine ($10^{-12}$ M and $10^{-8}$ M) for variable time periods (2, 4 and 6 hr). At the end of the incubation periods cells were lysed, proteins were extracted and probed for p53.

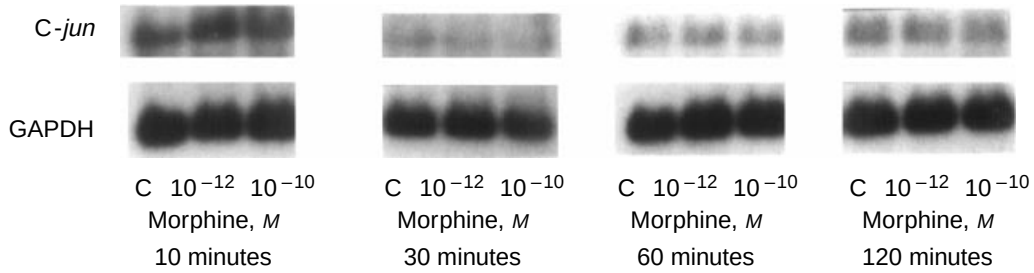


**Fig. 9. Effect of morphine on mRNA expression of c-*jun* on kidney fibroblasts.** The upper panel shows mRNA expression of c-*jun* by buffer (C) or morphine ($10^{-12}$ M and $10^{-10}$ M) treated kidney fibroblasts at indicated time periods. The lower panel shows expression of GAPDH by control and morphine treated fibroblasts at the indicated times.

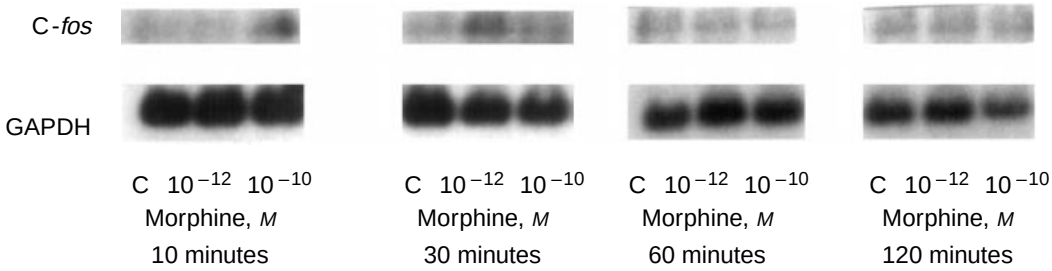


**Fig. 10. Effect of morphine on mRNA expression of c-*fos* on kidney fibroblasts.** The upper panel shows mRNA expression of c-*fos* by kidney fibroblasts after treatment with either buffer (C) or morphine ($10^{-12}$ M and $10^{-10}$ M) for 10, 30, 60 and 120 minutes. The lower panel shows expression of GAPDH by kidney fibroblasts at the indicated times.

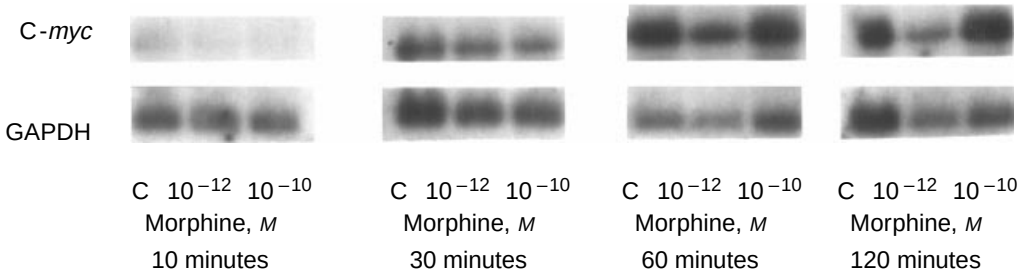


**Fig. 11. Effect of morphine on mRNA expression of c-*myc* by kidney fibroblasts.** The upper panel shows mRNA expression of c-*myc* by buffer (C) or morphine ($10^{-12}$ M and $10^{-10}$ M) treated fibroblasts at the indicated time periods. The lower panel shows GAPDH expression by buffer or morphine treated fibroblasts at the same time periods.

concentrations, morphine-induced cell suppression may partly attributed to the occurrence apoptosis. First, apoptotic cells do not proliferate and may not contribute to the proliferating cell pool. Second, cell numbers decline because many of the apoptotic cells may detach from the substrate and may not be available at the time of cell counting.

Apoptosis is a highly regulated form of cell death and is distinct from necrosis. Cells that die by apoptosis go through two sequential cycles such as commitment and execution [25, 26]. The duration of the commitment phase may be quite variable ranging from minutes to days, whereas the execution phase is relatively constant in duration ranging from 15 to 60 minutes. Cells during the commitment phase have normal morphology, whereas cells show classical features of apoptosis during the execution phase [25, 26]. Several genes have been implicated to regulate apoptosis [26–28]. In humans, these include *p53*, c-*Myc*, and *Bcl*-2. The gene p53 has been demonstrated to play an important role in apoptosis induced by DNA damage [29, 30]. It is one of the genes most

**Table 1.** Densitometric analysis of Northern blots

| | 10 min Morphine | | | 30 min Morphine | | | 60 min Morphine | | | 120 min Morphine | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | $10^{-12}$ M | $10^{-10}$ M | Control | $10^{-12}$ M | $10^{-10}$ M | Control | $10^{-12}$ M | $10^{-10}$ M | Control | $10^{-12}$ M | $10^{-10}$ M |
| A | 0.09 ± 0.01 | 0.13 ± 0.04 | 0.21 ± 0.05 | 0.17 ± 0.002 | 0.21 ± 0.05 | 0.24 ± 0.002 | 0.36 ± 0.01 | 0.37 ± 0.07 | 0.37 ± 0.07 | 0.19 ± 0.01 | 0.18 ± 0.02 | 0.37 ± 0.04 |
| B | 0.20 ± 0.01 | 0.34 ± 0.01 | 0.29 ± 0.07 | 0.19 ± 0.02 | 0.20 ± 0.02 | 0.23 ± 0.05 | 0.25 ± 0.01 | 0.19 ± 0.01 | 0.17 ± 0.03 | 0.18 ± 0.01 | 0.19 ± 0.03 | 0.12 ± 0.03 |
| C | 0.13 ± 0.0 | 0.11 ± 0.01 | 0.09 ± 0.11 | 0.13 ± 0.002 | 0.31 ± 0.09 | 0.88 ± 0.42 | 0.60 ± 0.04 | 0.57 ± 0.05 | 0.65 ± 0.17 | 1.40 ± 0.02 | 2.15 ± 0.27 | 2.27 ± 0.25 |

Densitometric analysis (mean ± SEM, $N = 3$) of the ratios between c-*fos* (A)/c-*jun* (B)/c-*myc* (C) and GAPDH expression was performed for control as well as morphine ($10^{-12}$ M and $10^{-10}$ M) treated cells at variable time periods (10, 30, 60 and 120 minutes).

commonly mutated in human malignancies [29]. The product of the p53 gene encodes a putative transcription factor and interacts with a variety of proteins involved in the progression of the cell cycle [29, 30]. We hypothesize that morphine-induced enhanced synthesis of p53 may have mediated fibroblast apoptosis.

Renal interstitial scarring is a major component of heroin associated nephropathy. We hypothesize that drug addicts in the beginning use a lesser amount of morphine that may initiate the proliferation of kidney fibroblasts as shown by physiological concentrations of morphine. However, gradually, drug addicts develop a tolerance to morphine, and to get a similar effect they may have to increase the dosage. Morphine at higher concentrations may lead to the suppression of fibroblast proliferation by promoting apoptosis. Hence, in due time morphine has the potential to induce a lesion characterized by initial hyperplasia and subsequent hypoplasia of interstitial cells.

The majority of patients with human immunodeficiency virus (HIV)-associated nephropathy are intravenous drug abusers [31, 32]. Drugs have been implicated to contribute to the development of HIV-associated nephropathy [31, 32]. Cocaine has been reported to enhance replication of the HIV virus [33]. Morphine activates peripheral mononuclear cells to secrete TGF-$\beta$ [34]. The latter has been demonstrated to enhance the expression of HIV-1 genes in cultured mesangial cells [35]. Renal interstitial scarring is a predominant component of HIV-associated nephropathy. It is likely that drugs such as morphine may also be contributing to the development as well as progression of renal interstitial scarring in patients with HIV-associated nephropathy.

Recently, Friedman and Rao suggested that the contaminants in street heroin contributed to the increased incidence of heroin-associated nephropathy in the 1980s and earlier periods in the metropolitan area of New York City [36]. However, these authors' stipulation was based on two circumstantial observations (noted in 1990s): (*1*) there was a decrease in the incidence of heroin-associated nephropathy in the metropolitan area, and (*2*) there was an availability of contaminant-free (relatively pure) street heroin. These authors linked these two observations and inferred that contaminants in the street heroin were responsible for the previously reported incidence of heroin-associated nephropathy in the metropolitan area [36]. Since there has been an increase in the incidence (of similar magnitude as reported for heroin nephropathy in 1980s) of another glomerulopathy (HIV-associated nephropathy) in the intravenous drug abuser population of the New York City metropolitan area, we differ from the interpretation of these authors [36]. We speculate that intravenous drug abusers are susceptible for developing renal lesions as well as HIV infection. Because of this dual susceptibility, this segment of the New York City area population is now more likely to develop HIV-associated nephropathy. We are not suggesting that HIV has not contributed to the development of this glomerular lesion. There are enough instances where HIV infection has been demonstrated to be solely responsible for the development of HIV-associated nephropathy [37]. However, this must not dampen our enthusiasm to evaluate the role of heroin in the development of the renal lesion.

We conclude that morphine directly modulates the proliferation of kidney fibroblasts and has a bimodal action. The present study provides a mechanistical insight into the development of renal interstitial scarring in patients with heroin addiction.

## ACKNOWLEDGMENTS

This work was supported by a grant 2RO1 DA 07653 from National Institute on Drug Abuse. This work was presented in the annual meeting of the College on Problems of Drug Dependence on June 17, 1997 in Nashville, Tennessee, USA.

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## APPENDIX

Abbreviations used in this article are: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FCS, fetal calf serum; HIV, human immunodeficiency virus; HRP, horseradish peroxidase; IGF-1, insulin-like growth factor; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; TCA, trichloroacetic acid; TE buffer, Tris and EDTA; TGF-$\alpha$, transforming growth factor-alpha; TUNEL, terminal deoxy transferase uridine triphosphate nick end-labeling.

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