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Effects of heparin on growth factor-induced mitogenic and proliferative responses of rat pulmonary artery smooth muscle cells[1]

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KEY WORDS heparin; platelet-derived growth factor; fibroblast growth factor; interleukin-1; vascular smooth muscle; cultured cells; rats

AIM: To investigate whether heparin inhibits growth factor-induced mitogenic and proliferative responses of the pulmonary arterial smooth muscle cells (PASMC). **METHODS**: Rat PASMC were cultured in medium 199 containing 10 % fetal bovine serum (FBS). Mitogenesis was monitored from [*methyl*-^{3}H] thymidine ([*methyl*-^{3}H] TdR) uptake and cell proliferation was monitored by cell counting. **RESULTS**: FBS (1 %), platelet-derived growth factor (PDGF, 50 $\mu g \cdot L^{-1}$), fibroblast growth factor (FGF, 50 $\mu g \cdot L^{-1}$) or interleukin 1α (IL-1α, 100 $ng \cdot L^{-1}$) alone induced both rat PASMC mitogenesis and proliferation. FBS (10 %) and the combination of FBS (1 %) with PDGF (50 $\mu g \cdot L^{-1}$), FGF (50 $\mu g \cdot L^{-1}$), or IL - 1α (100 $ng \cdot L^{-1}$) increased mitogenesis of rat PASMC. Heparin (100 $mg \cdot L^{-1}$) inhibited rat PASMC proliferation (28 % ± 6 %) and thymidine incorporation (27 % ± 7 %) induced by FBS (10 %), and also significantly inhibited cell proliferation 25 % ± 6 %, 27 % ± 7%, and 20 % ± 4%, and [*methyl*-^{3}H] TdR incorporation 23 % ± 7 %, 26 % ± 6 %, 20 % ± 6 % induced by FBS (1 %) in combination with PDGF (50 $\mu g \cdot L^{-1}$), FGF (50 $\mu g \cdot L^{-1}$), or IL-1α (100 $ng \cdot L^{-1}$) respectively. The PASMC viability was not affected by heparin. **CONCLUSION**: Proliferation and mitogenesis of rat PASMC induced by PDGF, FGF, and IL-1α was augmented by simultaneous exposure to FBS. Heparin produced an inhibition on the proliferation and mitogenesis of rat PASMC caused by these growth factors.

Several mediators played an important role in the development of pulmonary hypertension in animal models. There was evidence for involvement of mitogens, including interleukin-1[1], endothelin-1[2,3], tumor necrosis factor α (TNF-α)[4], and platelet-derived growth factor[5] in the development of pulmonary vascular remodeling in chronic pulmonary hypertension. Interleukin-1 was a major cytokine derived from macrophages and monocytes[6] and IL-1α receptor antagonist prevented monocrotaline-induced pulmonary vascular remodeling in rats[1]. IL-1 interacted with basic fibroblast growth factor to amplify the proliferation of primary rat aortic smooth muscle cells, an effect that might be important in vascular smooth muscle cell proliferation following[7].

Mononuclear cells, including monocyte-*macrophages and neutrophils were present in the* perivascular cellular infiltrate[8]. These cells, when activated, could release mitogens that promote vascular media remodeling[9]. Heparin produced partial reversal on pulmonary hypertension including both increased pulmonary artery pressure and vascular remodeling induced by hypoxia in guinea pig[6]. To date, no information is available concerning the potential effects of heparin on growth factor-induced mitogenesis and proliferation of the pulmonary arterial smooth muscle cells (PASMC). The objective of the present investigation was to determine the effects of heparin on mitogenesis and proliferation of PASMC induced by several mitogenic factors with a suspected involvement in the development of vascular remodeling associated with pulmonary hypertension.

MATERIALS AND METHODS

Materials Plastic ware was obtained from Corning Costar Corp USA. Tissue culture plates (96-well, flat bottom) were

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obtained from Falcon (Becton Dickinson Labware, Lincoln Park NJ). All chemicals used were of analytical grade or higher. BSA, fetal bovine serum, amphotericin B, trichloroacetic acid, indomethacin, Giemsa stain, $NaHCO_3$, fibroblast growth factor (from bovine pituitary glands), collagenase type class XI, trypsin, A-2547 (monoclonal anti α-smooth muscle actin, clone 1A4), Fc and Fab fragment specific FITC-conjugated anti-mouse IgG antibodies, benzylpenicillin, streptomycin, HEPES, M 199, heparin, and PBS were all from Sigma Chemical Co. *L*-glutamine from Life Technologies, (Paisley, UK); [*methyl*-^{3}H]TdR (37 PBq·mol^{-1}) from ICN Radiochemicals, Biomedical, Inc; platelet-derived growth factor, interleukin-1α (recombined human, *E coli*-derived) were obtained from R&D Systems, (Minneapolis, MN, USA). All other compounds including tissue culture were obtained from Sigma.

Culture of rat PASMC Isolation and culture of rat PASMC were made[11]. Wistar rats weighing 334 ± *s* 35 g were from Animal Resource Center, King's College London. The pulmonary artery was dissected free of connective tissue under aseptic conditions under a dissection microscope. Endothelium was removed from the luminal surface by scraping with a scalpel blade. Sections of pulmonary artery were incubated with collagenase type XI (2.5 g·L^{-1}) at 37 ℃ for 2.5 h. The tissues were washed in Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) and cut into 1 – 2 mm^3 pieces. Smooth muscle cells were obtained from plated explants[10]. The explants were seeded in a 25-cm^2 tissue culture flask containing medium 199 (M199) supplemented with *L*-glutamine 2 mmol·L^{-1}, fetal bovine serum (FBS, 10 % vol/vol), benzylpenicillin 100 kU·L^{-1}, streptomycin 0.1 g·L^{-1} and amphotericin B 0.2 g·L^{-1}, at pH 7.3 ~ 7.4, gassed with 95 % air + 5 % CO_2 in a humidified atmosphere at 37 ℃. The medium was changed every 3 d. Cells migrated from the explants after 2 wk. When cells migrating from the explant reached 10% of confluence, the cells were washed in PBS and incubated with 0.05 % trypsin plus edetic acid 5.3 mmol·L^{-1} in PBS for 5 min[10]. Cells were used at passage 3 – 6.

Characterization of PASMC PASMC were subjected to immunostaining with anti-smooth muscle α-actin[10], followed by imaging with fluorescein isothiocyanate (FITC) conjugated secondary antibodies specific for Fc and Fab portions of the primary anti-α-actin antibody.

Incorporation of [*methyl*-^{3}H]TdR Rat PASMC were plated (3×10^3 cells per well) in M 199 containing 10 % FBS. Six replicates were used for each experiment. At 24 h before stimulation, the medium was replaced with FBS-free medium (M 199 supplemented with bovine serum albumin [BSA, 0.5 % wt/vol]) to arrest cell growth. Potential inhibitors or their vehicles were added 30 min before mitogenic stimulation for 48 h. [*methyl*-^{3}H]TdR (37 GBq·L^{-1}, 10 μL/well) was added in the last 24 hours of mitogenic stimulation. Cells were washed in PBS to remove extracellular [*methyl*-^{3}H]TdR. Acid soluble radioactivity was removed by 30-min treatment with 5 % trichloroacetic acid at 4 ℃, followed by washing with 95 % ethanol. The remaining acid insoluble material was solubilized by 30-min incubation with 2 % Na_2CO_3 in NaOH 0.1 mol·L^{-1}[9]. The radioactivity was determined by liquid scintillation counting.

Evaluation of cell proliferation Cells were plated with the starting density of 100 cells/well. Cells were arrested 24 h before addition of mitogen. Potential inhibitor was added 30 min before mitogen, which was conducted for 3 d before Giemsa stain[10]. Cell viability was monitored by trypan blue exclusion[9].

Statistical analysis Comparison of cell densities within experiments was conducted with unpaired *t* test.

RESULTS

Rat PASMC Cells at confluence displayed a non-parallel distribution of cell growth with regions of multilayered cells ("Hill and Valley"). Cells stained positive for α-actin exhibited discrete actin fibers. Loss of cell viability was not detected at heparin 1 – 100 mg·L^{-1}.

Mitogenic and proliferative responses to mitogens in rat PASMC PDGF(50 μg·L^{-1}) and FGF (50 μg·L^{-1}) increased [*methyl*-^{3}H]TdR uptake into rat PASMC compared with control ($P < 0.05$). FBS (10 %) and the combination of FBS (1 %) with PDGF (50 μg·L^{-1}), FGF (50 μg·L^{-1}), and IL-1α (100 ng·L^{-1}) significantly increased incorporation of [*methyl*-^{3}H]TdR into rat PASMC by 492 % ± 57 %, 308 % ± 34 %, 272 % ± 35 %, and 376 % ± 66 % compared with FBS (1 %), or PDGF (50 μg·L^{-1}), FGF (50 μg·L^{-1}), IL-1α (100 ng·L^{-1}) alone, respectively (Tab 1).

Effects of heparin on growth factor-induced mitogenesis and proliferation Heparin (100 mg·L^{-1}) inhibited proliferation of rat PASMC in response to FBS (10 %), and PDGF (50 μg·L^{-1}), FGF (50 μg·L^{-1}) or IL-1α (100 ng·L^{-1}) in the presence of FBS (1 %) ($P < 0.05$, Tab 2).

Heparin (30 mg·L^{-1}) inhibited proliferation of rat PASMC in response to FBS (10 %), and PDGF (50 μg·L^{-1}), FGF (50 μg·L^{-1}) in the presence of FBS (1 %) ($P < 0.05$, Tab 2). Heparin exhibited similar potency at inhibiting these growth factor-induced mitogenesis and proliferation in a concentration-dependent manner (Tab 2, 3), and there existed a close correlation between inhibition of

Tab 1. Incorporation of [*methyl*-^{3}H] thymidine into DNA of rat PASMC in the presence of growth factors. n = 3 rats (6 wells for each treatment in each experiment), $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control; ^fP, ^iP, ^lP, $^oP < 0.01$ *vs* 1 % FBS, PDGF, FGF, or IL-1 alone, respectively.

Treatment	[*methyl*-^{3}H]Thymidine incorporation into DNA Bq/well
FBS free medium	590 ± 63
1% FBS	610 ± 69^a
PDGF 50 μg·L^{-1}	1 060 ± 130^c
FGF 50 μg·L^{-1}	1 130 ± 120^c
IL-1α 100 ng·L^{-1}	700 ± 110^a
1 % FBS + PDGF 50 μg·L^{-1}	3 270 ± 360^{cf}
1 % FBS + FGF 50 μg·L^{-1}	3 070 ± 400^{ci}
1 % FBS + IL-1α 100 ng·L^{-1}	2 630 ± 460^{cl}
10 % FBS	3 000 ± 350^{co}

Tab 2. Inhibitory effect of heparin on mitogenic factor-induced incorporation of [*methyl*-^{3}H] TdR into rat PASMC. n = 3 rats (6 replicates for each treatment in each experiment), $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* control.

Treatment/ mg·L^{-1}	10 % FBS	[*methyl*-^{3}H]Thymidine uptake (Bq/well) 1 % FBS + PDGF 50 μg·L^{-1}	1 % FBS + FGF 50 μg·L^{-1}	1 % FBS + IL-1α 100 ng·L^{-1}
Heparin free	3 000 ± 340^a	3 300 ± 370^a	3 300 ± 420^a	2 500 ± 440^a
Heparin 1	2 990 ± 420^a	3 200 ± 470^a	3 200 ± 370^a	2 700 ± 540^a
Heparin 3	2 700 ± 570^a	3 200 ± 410^a	3 300 ± 470^a	2 500 ± 420^a
Heparin 10	2 400 ± 550^a	2 800 ± 550^a	2 800 ± 530^a	2 600 ± 390^a
Heparin 30	2 200 ± 460^b	2 600 ± 450^b	2 600 ± 340^b	2 400 ± 320^a
Heparin 100	2 200 ± 610^b	2 500 ± 790^b	2 400 ± 460^b	2 000 ± 360^a

mitogenesis and proliferation caused by the 4 groups of mitogens over the entire concentration range (r = 0.989, 0.952, 0.985, 0.800, respectively, Fig 1).

DISCUSSION

Heparin effectively inhibited the mitogenic and proliferative response to a variety of growth factors in the presence of low serum concentration, including PDGF, FGF, and IL-1α with similar potency. This suggests that heparin does not act by blockade of a specific membrane receptor, but rather via a more generalized route. A similar conclusion was drawn from examination of anti-mitogenic effect of heparin on *bovine tracheal smooth muscle cells in culture*[10]. The inhibitory action of heparin on the mitogenic and proliferative responses of PAMSC is not secondary to cell death, since cell viability was unaffected at concentrations of heparin which decreased cell proliferation. Thus the current findings are consistent with the direct inhibition of heparin on proliferation and mitogenesis of PASMC and this action being responsible, at least in part, for the ability of heparin *in vivo* to inhibit pulmonary vascular remodeling.

Endothelin-1 plays a major role in pulmonary vasoconstriction and pulmonary vascular remodel-

Tab 3. Inhibitory effect of heparin on mitogenic factor-induced proliferation of rat PASMC. n = 3 rats (6 replicates for each treatment in each experiment), $\bar{x} \pm s$, data are direct cell counting. $^aP > 0.05$, $^bP < 0.05$ *vs* control.

Treatment/ mg·L^{-1}	Cell number (cells/well) 10 % FBS	1 % FBS + PDGF 50 μg·L^{-1}	1 % FBS + FGF 50 μg·L^{-1}	1 % FBS + IL-1α 100 ng·L^{-1}
Heparin free	480 ± 89	490 ± 63	450 ± 75	420 ± 77
Heparin 1	440 ± 105^a	430 ± 115^a	460 ± 70^a	420 ± 75^a
Heparin 3	430 ± 95^a	460 ± 98^a	450 ± 65^a	390 ± 94^a
Heparin 10	360 ± 99^a	410 ± 102^a	390 ± 95^a	380 ± 67^a
Heparin 30	320 ± 55^b	370 ± 63^b	360 ± 785^a	330 ± 97^a
Heparin 100	310 ± 77^b	360 ± 60^b	320 ± 66^b	320 ± 78^b

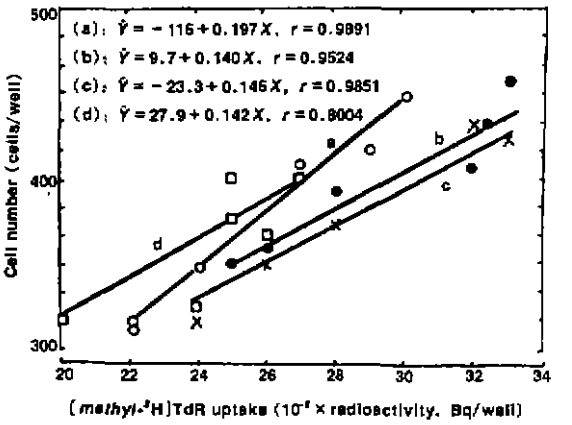

Fig 1. Correlation between heparin-induced inhibition of mitogenesis and proliferation caused by 10 % FBS (a), and PDGF 50 μg·L^{-1} (b), FGF 50 μg·L^{-1} (c) or IL-1α 100 ng·L^{-1} (d) in the presence of 1 % FBS.

ling[5]. PDGF is, however required for the full expression of pulmonary arterial smooth muscle cell growth-promoting activity of ET-1[5]. It has been reported that hypoxia stimulated the expression of PDGF and PDGF played an important role in the pulmonary vascular remodeling of hypoxic pulmonary hypertension[1,2]. As demonstrated in the present investigation, PDGF is a proliferative agent in rat pulmonary artery smooth muscle cell lines and others demonstrated that PDGF increased the expression of a variety of immediate-early gene[12]. By Northern analysis, it was found that PDGF A- and B-chain products may be coordinately and sequentially involved in hypoxic pulmonary vascular remodeling[12]. The present study further demonstrated that the proliferation and mitogenesis of rat PASMC induced by PDGF, FGF, and IL-1 were augmented by simultaneous exposure to FBS.

In conclusion, the direct inhibitory action of heparin on PASMC proliferation and mitogenesis suggested a potential mechanism contributing to heparin inhibition of pulmonary vascular remodeling. Clearly further investigation is required to fully understand the potential activities of heparin and the mechanisms by which it may inhibit the development of pulmonary vascular remodeling.

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肝素对生长因子诱导的大鼠肺动脉平滑肌细胞分裂和增殖的影响[1]

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关键词 肝素; 血小板衍化生长因子; 成纤维细胞生长因子; 白细胞介素-1; 血管平滑肌; 培养的细胞; 大鼠

目的: 探讨肝素是否能抑制生长因子诱导的大鼠肺动脉平滑肌细胞(PASMC)分裂和增殖. **方法**: 应用含 10% FBS 的 M-199 培养液培养大鼠 PASMC. 细胞分裂及细胞增殖分别用[*methyl*-^{3}H]TdR和细胞计数监测. **结果**: FBS (10 %), 以及 FBS (1 %)与 PDGF (50 $\mu g \cdot L^{-1}$), FGF (50 $\mu g \cdot L^{-1}$), 或 IL-1α (100 $ng \cdot L^{-1}$)联合应用均能增加大鼠 PASMC 分裂. 肝素(100 $mg \cdot L^{-1}$)抑制 10 % FBS 诱导的大鼠 PASMC 增殖(28 % ± 6 %)和胸腺嘧啶摄取反应(27 % ± 7 %), 抑制 FBS (1 %)与 PDGF (50 $\mu g \cdot L^{-1}$), FGF (50 $\mu g \cdot L^{-1}$), 或 IL-1α (100 $ng \cdot L^{-1}$)联用诱导的大鼠 PASMC 增殖(25 % ± 6 %, 27 % ± 7 %, 20 % ± 4 %), 以及胸腺嘧啶摄取反应(23 % ± 7 %, 26 % ± 6 %, 20 % ± 6 %). **结论**: 肝素抑制生长因子诱导的大鼠 PASMC 的分裂与增殖.