THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 22, Issue of May 28, pp. 23574–23579, 2004

Printed in U.S.A.

Butyric and Retinoic Mixed Ester of Hyaluronan

A NOVEL DIFFERENTIATING GLYCOCONJUGATE AFFORDING A HIGH THROUGHPUT OF CARDIOGENESIS IN EMBRYONIC STEM CELLS*

Received for publication, February 20, 2004, and in revised form, March 16, 2004
Published, JBC Papers in Press, March 24, 2004, DOI 10.1074/jbc.M401869200

Carlo Ventura‡§, Margherita Maioli¶, Yolande Asara¶, Daniela Santoni¶, Ignazio Scarlata‖, Silvia Cantoni‖, and Alberto Perbellini‡

From the ‡Laboratory of Molecular Biology and Stem Cell Engineering, National Institute of Biostructures and Biosystems, University of Bologna, I-40138 Bologna, Italy, the ¶Department of Biomedical Sciences, University of Sassari, I-07100 Sassari, Italy, and the ‖Department of Biochemistry, Biophysics, and Chemistry of Macromolecules, University of Trieste, I-34127 Trieste, Italy

Embryonic stem (ES) cells can differentiate into specialized cells, including cardiac myocytes, but the efficiency is typically low and the process is incompletely understood. Achieving a high throughput of cardiogenesis from pluripotent cells is therefore a major requirement for future approaches in cardiac cell therapy. Here, we developed a novel ester of hyaluronan linked to both butyric and retinoic acid (HBR), coaxing pluripotent ES cells into a cardiogenic decision. In mouse ES cells, HBR remarkably increased the expression of *GATA*-4 and *Nkx-2.5*, acting as cardiac lineage-promoting genes in different animal species, including humans. HBR also enhanced prodynorphin gene expression and the synthesis and secretion of dynorphin B, an endorphin playing a major role in ES cell cardiogenesis. These effects occurred at the transcriptional level. HBR also primed the expression of cardiac-specific transcripts and highly enhanced the yield of spontaneously beating ES-derived cardiomyocytes. These results demonstrate the potential for chemically modifying the gene program of cardiac differentiation in ES cells without the aid of gene transfer technologies and may pave the way for novel approaches in tissue engineering and myocardial regeneration.

Embryonic stem (ES)[1] cells have been recently proposed as a renewable source of donor cardiomyocytes substituting for the irreversible cell loss in adult hearts damaged by acute myocardial infarction or hereditary cardiomyopathies (1, 2). Because the rescuing potential of ES cells is limited by the fact that ES-derived cardiomyocytes withdraw early from the cell cycle (1, 3), the development of strategies affording high throughput of cardiogenesis from pluripotent cells would have obvious therapeutic potential. However, overexpression of cardiogenic genes by vector-mediated gene transfer is a cumbersome approach that may perturb normal homeostasis in both ES cells and recipient tissues and is not readily envisionable in humans.

Here, we developed hyaluronan monoesters with retinoic acid alone (HR) and its mixed esters with retinoic and butyric acid (HBR) and provided evidence that these compounds act as novel differentiating agents eliciting a remarkable increase in the yield of cardiomyocytes derived from mouse ES cells. A number of interrelated observations prompt a rationale behind the synthesis of the novel glycoconjugate HBR. From the earliest observable stage of mouse heart development, the hyaluronan receptor CD44 is highly expressed by presumptive cardiogenic cells and by the fused endothelial tubes and surrounding muscular epimyocardium within the early embryonic heart (4). Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of mouse epithelium into mesenchyme (5); CD44 induction by hyaluronan has also been documented during ES cell differentiation (6). Within this context, hyaluronan can be translocated into various cell types via a receptor-mediated endocytosis because it is consistently detected in close association with nuclear heterochromatin (7, 8). Moreover, intracellular hyaluronan-binding molecules (hyaladherins) translocate to the nucleus upon mitogenic stimulation (9), serving as substrates or activators for MAP kinases (10), or have been shown to represent vertebrate homologues of proteins involved in splicing or cell cycle regulation (11, 12). These findings suggest that hyaluronan that is taken up may regulate cellular events from within subcellular compartments and may also act as a carrier for internalization of hyaluronan-grafting synthetic compounds. An interference of retinoic acid (RA) signaling with cardiac differentiation resulted from the finding that inactivation of the RXRα gene and vitamin A deficiency caused embryonal death owing to cardiac hypoplasia and ventricular chamber defects (13). Dramatic abnormalities in heart development were also obtained by combining mouse strains with mutant RAR and RXR subtypes (14, 15). Moreover, *in vitro* studies demonstrated that all-*trans* RA increased the efficiency of cardiogenic differentiation in ES cells (16). Concerning butyrate, its histone deacetylase inhibitory action alters chromatin structure, ultimately increasing transcription factor accessibility to target cis-acting regulatory sites (17).

* This work was supported by Ministero dell'Istruzione, dell'Università e della Ricerca (Fondo Integrativo Speciale per la Ricerca-2001), and Ministero della Sanità (Attività di Ricerca Finalizzata-2002), Italy, the Center for Biotechnology Development and Biodiversity Research, University of Sassari, Italy, and Sintofarm S.p.A. (Guastalla, Reggio Emilia), Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Laboratory of Molecular Biology and Stem Cell Engineering, National Institute of Biostructures and Biosystems, University of Bologna, S. Orsola-Malpighi Hospital, Institute of Cardiology, Pavilion 21, Via Massarenti N. 9, Bologna 40138, Italy. Tel.: 39-051-6363605; Fax: 39-051-344859; E-mail: cvent@libero.it.

[1] The abbreviations used are: ES, embryonic stem; DMF, *N*,*N*-dimethylformamide; EBs, embryoid bodies; LIF, leukemia inhibitory factor; PKC, protein kinase C; MHC, α-myosin heavy chain; DS, degree of substitution; BU, butyric acid; RA, retinoic acid; HA, hyaluronan; HB, HA monoesters with BU alone; HR, HA monoesters with RA alone; HBR, HA mixed esters with BU and RA; M_w, weight average molecular weight; TBA, tetrabutylammonium.

Accordingly, basic helix-loop-helix transcription factors interact with transcriptional coactivators possessing histone acetyltransferase activity (18) and histone deacetylase inhibitors have been reported to enhance RXR/RAR heterodimer action, promoting crucial developmental pathways in pluripotent cells (19, 20).

EXPERIMENTAL PROCEDURES

Preparation of Tetrabutylammonium Hyaluronate (HA-TBA)—HA-TBA was synthesized by recycling a 5% w/w solution in Milli-Q distilled water (Millipore) of sodium hyaluronate (Bioiberica), whose weight-average molecular weight (M_w) was 85,000, through Amberlite IR-120 (plus) ion exchange resin in TBA form at room temperature for 24 h. The collected HA-TBA solution was finally freeze-dried. Amberlite IR-120 (plus) ion exchange resin in acidic form (Aldrich) was previously converted into TBA form by treating with a 40% w/w aqueous solution of TBA hydroxide (Fluka) that was recycled in a jacket column at 40 °C for 4 days. The resin in TBA form was then washed with distilled water to reduce the pH value below 10.

Synthesis of Two Different HBR—HA-TBA (5 g; $8.1 \cdot 10^{-3}$ eq, *i.e.* molar equivalents, *i.e.* moles of dimeric repeating units) was dissolved in 40% w/w aqueous TBA hydroxide (5.2 ml; $7.8 \cdot 10^{-3}$ eq). The solution was then frozen and freeze-dried. Hereafter, all reactions were carried out at room temperature under magnetic stirring, under nitrogen flux, and by sheltering from light. The lyophilized TBA alcoholate of HA-TBA and all-*trans* RA (2.4 g; $8.0 \cdot 10^{-3}$ eq) (BASF) were separately dissolved in dry *N*,*N*-dimethylformamide (DMF) (300 and 20 ml, respectively) (Fluka). Meanwhile, dry DMF (1 ml; $1.3 \cdot 10^{-2}$ eq) and oxalyl chloride (1 ml; $1.1 \cdot 10^{-2}$ eq) (Fluka) were added to dry diethylether (5 ml) (Fluka). After a few minutes, the RA solution in DMF was added dropwise onto the obtained white solid (dimethylchloroformamidinium chloride) during 20 min. After 1 h, the clear deep red solution of retinoyl chloride was added by a dropping funnel at a flow rate of 1 ml/minute to the solution of TBA alcoholate of HA-TBA. The reaction mixture was kept in the same conditions as above for 17 h. The dark brown solution was then subdivided in two exactly equal fractions, called A and B. A was concentrated to about one third of the original volume by using a rotary film evaporator under reduced pressure. The polymer was precipitated into three volumes of diethylether and recuperated by suction filtration. The dark brown amorphous precipitate was dissolved again in dry DMF (100 ml), and triethylammine (300 μl; $2.2 \cdot 10^{-3}$ eq) (Aldrich) was then added to neutralize the acidic reagents. Butyrylation was carried out overnight by addition of butyric anhydride (640 μl; $3.9 \cdot 10^{-3}$ eq) (Fluka) and 4-(dimethylamino)pyridine (2 g; $1.6 \cdot 10^{-2}$ eq) (Fluka) to each one of the fractions. The raw products were precipitated from both A and B into 3 volumes of diethyl ether, recovered by suction filtration, washed several times with diethylether and acetone, and, lastly, dried under vacuum. After dissolution of the dry solids in distilled water and neutralization with 0.1 M aqueous NaOH, the polymers were purified by dialysis against Milli-Q distilled water, using a cellulose membrane tubing (Visking) with a molecular weight cut-off in the range of 12,000–14,000, and then by recycling the retained solutions through Amberlite IR-120 (plus) ion exchange resin in sodium form (30 ml; $5.7 \cdot 10^{-2}$ eq) (Aldrich) for 1 h. Two butyric and retinoic mixed esters of sodium hyaluronate (Fig. 1) were ultimately obtained as lyophilized solids by freeze-drying from A (0.8 g; $1.7 \cdot 10^{-3}$ eq; 21% yield) and B (1.2 g; $2.7 \cdot 10^{-3}$ eq; 33% yield). The products HBR were characterized by spectroscopy of proton nuclear magnetic resonance (^{1}H NMR). The spectra, recorded in deuterated dimethyl sulfoxide (Me_2SO-d6) at 500 MHz on a Varian Inova 500 spectrometer, showed the signals due to the protons of the polysaccharide and to all the protons of RA. From evaluation of the chemical shifts related to the signals due to RA, RA was confirmed to keep its all-*trans* isomeric form. The signals that confirmed the presence of butyrate residues (δ 0.90, 1.62, 2.39 ppm) were detected as well; they allowed the determination of the degree of substitution with butyric acid (DS_{BU}), which were 0.94 and 0.45 in the cases of A and B, respectively. The DS with retinoic acid (DS_{RA}) (0.046 for A and 0.066 for B) were determined by comparison of the UV-visible absorption spectra (Varian UV-VIS CARY 3E spectrophotometer) at the absorbance maximum (λ_{max} = 359 nm in Me_2SO) of standard RA solutions with those of HBR solutions (in the presence of 0.5% trifluoroacetic acid) at known concentration.

Synthesis of an HR Having DS_{RA} = 0.12—The procedure was the same as described in the case of HBR for synthesis as far as retinoylation and for purification after butyrylation by doubling all the starting materials except HA-TBA (5 g; $8.1 \cdot 10^{-3}$ eq). A retinoic ester of sodium hyaluronate (Fig. 1, formula with $y = 0$) was finally obtained as lyophilized solid by freeze-drying (1.7 g; $3.9 \cdot 10^{-3}$ eq; 48% yield). As for HBR, the product HR was characterized by ^{1}H NMR spectroscopy, which revealed the presence of signals attributable to residues of retinoate, and by UV-VIS spectrophotometry, which allowed the determination of the DS_{RA}, which was 0.12.

Synthesis of an HB Having DS_{BU} = 0.062—The following reaction was carried out at room temperature, under mechanical stirring, and under nitrogen flux. HA-TBA (55 g; $8.9 \cdot 10^{-2}$ eq) was completely dissolved in dry DMF (3 liters). The acylating agent was prepared by mixing butyric anhydride (4 ml; $2.45 \cdot 10^{-2}$ eq) and (dimethylamino)pyridine (3 g; $2.46 \cdot 10^{-2}$ eq) in dry DMF (200 ml). The latter solution was added dropwise to the former one during 25 min, and the reaction was continued for a further 65 min. The TBA salt of HB was converted into the correspondent sodium salt by addition of distilled water (~6 liters), acidification with 0.1 M aqueous HCl, and neutralization with 1 M aqueous NaOH. The polymer was purified as described in the case of HBR by dialysis against Milli-Q distilled water (14 times with 30 liters) and then by ion exchange with Amberlite IR-120 (plus) resin (500 ml; 0.95 eq). A butyric ester of sodium hyaluronate (Fig. 1, formula with $z = 0$) was finally obtained as lyophilized solid by freeze-drying (34 g; $8.4 \cdot 10^{-2}$ eq; 94% yield). As for HBR, the product HB was characterized by ^{1}H NMR spectroscopy, which revealed the presence of signals attributable to residues of butyrate and which allowed the determination of the DS_{BU} that was 0.062.

Determination of the Molecular Weight of Hyaluronan Esters—Molecular weight was determined by high-performance size-exclusion chromatography (HP-SEC). The analysis conditions were as follows. Chromatograph: HPLC Jasco PU-880 with Rheodyne 9125 injector. Columns: TSK PWXL G6000, G5000, and G3000 (TosoHaas), temperature 40 °C, mobile phase 0.15 M NaCl, flow rate 0.8 ml/minute. Detectors: low angle laser light scattering CMX-100 (Chromatix); differential refraction index 410 (Waters), sensitivity 128×, temperature 32 °C. The products were dissolved in 0.15 M NaCl at a concentration of ~1.0 mg/ml and kept under stirring for 12 h. The solutions were filtered through 0.45-μm pore size filters (Millipore) and then injected (100 μl) into the chromatograph. The concentrations of the hyaluronan solutions were checked by the integral of the refractive index. The analysis allowed the determination of the weight-average molecular weight (M_w), the number-average molecular weight (M_n), and the distribution of molecular weight by using a broad standard calibration (based on sodium hyaluronate). The M_w of HBR and HR were determined to range between 10,000–30,000, whereas the M_w of HB was determined to range between 50,000–85,000.

ES Cell Culture—GTR1, a derivative of R1 ES cells (21) bearing the puromycin-resistance gene driven by the cardiomyocyte-specific α-myosin heavy chain promoter, were kindly provided by Dr. William L. Stanford (University of Toronto and Centre for Modeling Human Disease, Canada). Cardiac differentiation and puromycin selection of ES-derived cardiomyocytes were performed as previously described in detail (22). Briefly, cells were maintained in the undifferentiated state by culturing onto a layer of mitotically inactivated mouse embryo fibroblasts in the presence of knockout Dulbecco's modified Eagle's medium containing 15% fetal bovine serum, supplemented with a final concentration of 1000 units/ml leukemia inhibitory factor (LIF). Before embryoid bodies (EBs) can be made, subconfluent undifferentiated ES cells are harvested from feeder layers by trypsinization, transferred onto 0.1% gelatin-coated plates, and grown to about 70–80% confluence in the presence of LIF-supplemented knockout Dulbecco's modified Eagle's medium containing 15% fetal bovine serum. These cells were used as undifferentiated cells in each experiment. The undifferentiated state of cells cultured with LIF on both feeder layers and gelatinized plates was inferred from the high percentage of cells positively stained for alkaline phosphatase activity and from the lack of nestin-positive cells. To induce cardiac differentiation, cells were plated onto specialty plates (Costar ultra low attachment clusters) containing the culture medium lacking supplemental LIF. After 2 days of culture, the EBs were plated onto tissue culture dishes. When spontaneous contractile activity was noticed (7 days after LIF removal), puromycin (2 μg/ml) was added to eliminate non-cardiomyocytes and puromycin-selected cells were cultured for an additional 7 days. Analysis of myosin heavy chain (MHC) immunoreactivity revealed that at this stage cardiomyocytes comprised more than 99% of selected cells. EBs, collected at several stages after plating, as well as puromycin-selected cells were processed for gene expression and immunofluorescence analyses.

In selected experiments, GTR1 or parental R1 cells were also committed to neurogenesis or myogenesis as previously described (23). Briefly, for the neurogenic commitment EBs were cultured for 4 days on Costar ultra low attachment clusters containing the culture medium

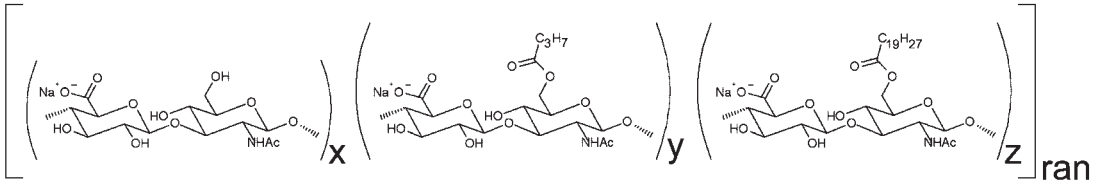

FIG. 1. **Chemical structure of hyaluronan esters.** The general formula of HA esters represents a random copolymer (*ran*) of three distinct dimeric repeating units, among which *x* are non-substituted, *y* are butyrylated (C_3H_7CO group) and *z* are retinoylated ($C_{19}H_{27}CO$ group). Let *n* be the sum of *x*, *y*, and *z*, namely the total number of disaccharide units in the polysaccharide. The DS_{BU} and DS_{RA} correspond to the ratio between *y* and *n* and between *z* and *n*, respectively. Obviously, for HR the DS_{BU} is 0 ($y = 0$), whereas for HB the DS_{RA} is 0 ($z = 0$).

lacking supplemental LIF in the presence of 0.5 μM RA. Then, cells were plated onto tissue culture dishes without RA and processed for the analysis of neurogenin-1 gene transcription after an additional 4 days. To elicit development into skeletal muscle cells, EBs were cultured for 4 days onto ultra low attachment clusters in the presence of the culture medium lacking supplemental LIF containing 30 nM sodium selenite, bovine serum albumin (0.19%), and 0.01 mg/ml transferrin. Afterward, cells were plated on tissue culture dishes and processed for the analysis of *MyoD* gene transcription after an additional 7 days.

Gene Expression—Total RNA extraction, reverse transcription, and PCR conditions were previously described (22, 24). Prodynorphin, *GATA-4*, and *Nkx-2.5* mRNAs were assessed by RNase protection assay as detailed elsewhere (22). Fragments of the main exon of mouse prodynorphin gene (424 bp), *GATA-4* (292 bp), or *Nkx-2.5* (414 bp) genes were inserted into pCRII-TOPO. Transcription of the plasmid linearized with ApaI, BamHI, or XbaI generated sense strands of prodynorphin, *GATA-4*, or *Nkx-2.5* mRNA, respectively, that were used to construct a standard mRNA curve. Transcription in the presence of [^{32}P]CTP of plasmids linearized with BamHI generated antisense strands of prodynorphin and *Nkx-2.5* mRNA, whereas transcription of plasmids linearized with XbaI produced an antisense strand of *GATA-4* mRNA.

Isolation of ES Cell Nuclei and Nuclear Run-off Transcription Assay—Isolation of nuclei and assessment of nuclear purity were performed as detailed elsewhere (22, 23). Only freshly isolated nuclei were used in each experiment. Nuclear run-off experiments were carried out as previously described (23). Briefly, nuclear RNA was isolated by using guanidine thiocyanate and acid phenol extraction, followed by purification on RNAMATRIX™. Equal counts of ^{32}P-labeled RNA (about $5 \cdot 10^6$ cpm) were then subjected to a solution hybridization RNase protection assay and were hybridized for 12 h at 55 °C in the presence of unlabeled antisense *GATA-4*, *Nkx-2.5*, prodynorphin, *MyoD*, or neurogenin-1 mRNA. To generate these cRNA probes, fragments of the main exon of mouse prodynorphin gene (424 bp), *GATA-4* (292 bp), *Nkx-2.5* (414 bp), *MyoD* (425 bp) or neurogenin-1 (also called neuroD3) (742 bp) genes were inserted into a pCRII-TOPO vector. Transcription of plasmids linearized with BamHI generated antisense strands of prodynorphin, *Nkx-2.5*, and *MyoD* mRNA, whereas transcription of plasmids linearized with XbaI produced an antisense strand of *GATA-4* and neurogenin-1 mRNA. Samples were then incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were recovered after phenol chloroform extraction and electrophoretically separated in a polyacrylamide non-denaturing gel. Autoradiographic exposure was for 48 h. ^{32}P-labeled nuclear RNA was also hybridized with unlabeled antisense cyclophilin mRNA synthesized from an NcoI-linearized pBS vector containing a 270-base pair fragment of plB15, a cDNA clone encoding for rat cyclophilin (23). Cyclophilin mRNA was utilized as a constant mRNA for control.

Immunofluorescence Analysis of ES-derived Cardiomyocytes—Puromycin-selected cells were treated with trypsin, and the resulting suspension was cultured at low density to permit visualization of individual cells. The cultures were fixed with 4% paraformaldehyde. MHC was assessed by the aid of the MF 20 mouse antimyosin monoclonal antibody (22). All microscopy was performed with a Bio-Rad Microradians confocal microscope. DNA was visualized with propidium iodide (1 μg/ml).

Identification of Dynorphin B-like Material—Immunoreactive dynorphin B was measured, as previously detailed (22, 24), by a radioimmunoassay procedure that utilized the 13 S antiserum raised against dynorphin B capable of recognizing the high molecular weight peptides cleaved from the prodynorphin precursor and containing dynorphin B in their sequences (22). Acetic acid extracts from undifferentiated or cardiac lineage-committed ES cells or pooled samples from their incubation media were processed by reverse-phase high-performance liquid chromatography. The collected fractions were radioimmunoassayed,

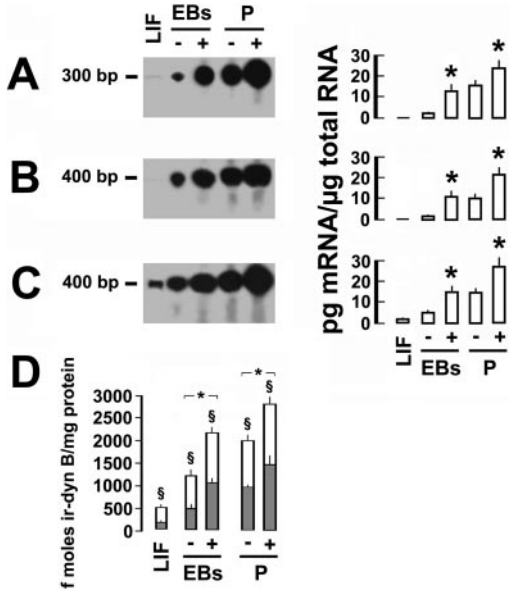

FIG. 2. **HBR primes cardiac lineage-promoting genes.** Following LIF removal, cells were treated without (−) or with (+) HBR (0.75 mg/ml). *LIF*, undifferentiated cells. *EBs*, embryoid bodies collected 5 days after LIF removal. *P*, ES-derived cardiomyocytes 4 days after puromycin addition. *A–C*, RNase protection analysis of *GATA-4*, *Nkx-2.5*, and prodynorphin mRNA, respectively. Equal amounts of total RNA (4 μg) from each sample were used in each RNase protection analysis. Autoradiographic exposure was for 2 days on Kodak X-Omat film with an intensifying screen. The *left* side of each panel reports the position of radiolabeled DNA markers, showing that the single protected fragments migrated with a molecular size comparable with *GATA-4* (292 bases), *Nkx-2.5* (414 bases), or prodynorphin (424 bases) mRNA. Averaged mRNA levels (mean ± S.E.; $n = 6$) are reported in the *right* part of each panel. —, non-detectable. *D*, immunoreactive dynorphin B (*ir-dyn B*) in cells (*gray bars*) or medium (*white bars*), mean ± S.E. ($n = 6$). *Asterisks with brackets*, significant difference (one-way analysis of variance, Newman Keul's test). §, significantly different from the values of the *gray bars*.

and the immunoreactivity was attributed to authentic dynorphin B by comparison with the elution position of a synthetic standard according to a previously described procedure (24).

Data Analysis—The statistical analysis of the data was performed by using a one-way analysis of variance followed by Newman Keul's test and assuming a p value <0.05 as the limit of significance.

RESULTS AND DISCUSSION

The glycoconjugates developed in the current investigation are esters between the hydroxyl groups of hyaluronan and the carboxyl group of butyric acid alone (HB), retinoic acid alone (HR), or the ones of both butyric and retinoic acids (HBR). The primary hydroxyl group in position 6 of the *N*-acetyl-D-glucosamine residues in the polysaccharide backbone is the most reactive toward esterification (Fig. 1).

During the study, several HB, HR, and HBR were synthesized to evaluate the relationship between degree of substitution (DS) and pharmacological effect. DS (Fig. 1) was considered as the number of the esterified OH groups for each

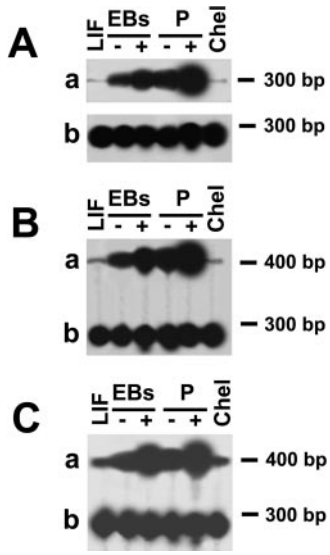

FIG. 3. **HBR acts at the transcriptional level.** After LIF withdrawal, cells were treated without (−) or with (+) HBR (0.75 mg/ml). Nuclei were from undifferentiated cells (*LIF*), EBs collected 5 days after LIF removal, or recovered 4 days (*P*) after puromycin addition. *Chel*, nuclei of cardiomyocytes derived from cells previously cultured with 0.75 mg/ml HBR in the presence of 5 μM chelerythrine following LIF removal and throughout 4 days of puromycin selection. *A–C*, *row a*, nuclear run-off analysis of *GATA-4*, *Nkx-2.5*, or prodynorphin genes, respectively. *Row b*, cyclophilin mRNA. Autoradiograms are representative of six separate experiments.

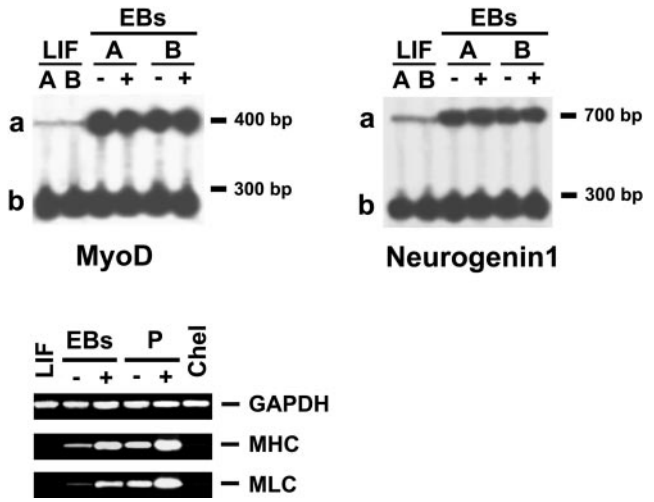

FIG. 4. **Effect of HBR on non-myocardial and cardiac-specific transcripts.** *Upper panel*, as described under "Experimental Procedures," after LIF withdrawal GTR1 ES cells (*A*) or the parental R1 ES cells (*B*) were committed to myogenesis or neurogenesis in the presence of defined media and treated in the absence (−) or presence (+) of HBR (0.75 mg/ml). Nuclei were isolated from undifferentiated cells (*LIF*) or cells committed to the selected lineages (*EBs*) and processed for the nuclear run-off analysis of the indicated transcripts. *Row a,* transcription of *MyoD* or neurogenin-1 genes. *Row b,* cyclophilin mRNA. The *right* side of each panel reports the position of radiolabeled DNA markers, showing that the single protected fragments migrated with a molecular size comparable with *MyoD* (425 bases), neurogenin-1 (742 bases), or cyclophilin (270 bases) mRNA. Autoradiograms are representative of six separate experiments. *Lower panel*, RT-PCR analysis of α-myosin heavy chain (*MHC*) and myosin light chain-2V (*MLC*) in *EBs* and *P*. Cell collection and treatment without (−) or with (+) HBR or with HBR plus chelerythrine (*Chel*) were as described in Figs. 2 and 3.

repeating unit of hyaluronic acid (GlcNAc-GlcUA dimer).

All the synthesized HBR exhibited a DS_{BU} ranging between 0.05–1.0, whereas the DS_{RA} was between 0.002–0.1. The DS_{BU}/DS_{RA} ratio was at least 6. The synthesized HR had a DS_{RA} ranging between 0.005–0.2. The synthesized HB had a DS_{BU} ranging between 0.004–0.8. The weight-average molecular weight (M_w) of these HA esters, referred to as the M_w of sodium hyaluronate, was determined by high-performance size-exclusion chromatography and ranged between 10,000–30,000 in the cases of HR and HBR or between 50,000–85,000 in HB.

In the earlier procedure that illustrated the synthesis of HB (25), we achieved solubility in DMF by preparing the symcollidinium salt of the D-glucuronic acid carboxylic functions of HA in water medium. Water was subsequently replaced with DMF but could not be totally removed; for this reason the acylating agent was partially hydrolyzed. In this novel synthetic methodology of HA esters, a TBA salt of HA-TBA was prepared; the good solubility of this salt in polar aprotic organic solvents such as *N*,*N*-dimethylformamide (26) allows synthesis in an anhydrous and homogeneous reaction system. To increase their nucleophylicity toward retinoylation, the activation of the hydroxyl groups of HA-TBA was carried out by the formation of alcoholates of TBA. Furthermore, treatment with such a strong base as TBA hydroxide has provoked a convenient reduction of the hyaluronan molecular weight and dimension by hydrolysis, thus enhancing its bioavailability and solubility and decreasing the viscosity of its solutions. The esterification (acylation) reactions were accomplished by means of activated reactive forms of butyric acid, precisely the *N*-acylpyridinium salt (27) between butyric anhydride and 4-(dimethylamino)pyridine, and of retinoic acid, specifically its acyl (retinoyl) chloride (28). Retinoylation should take place before butyrylation because of the longer chemical kinetics of the former reaction.

Here, HBR was administered to GTR1 cells, a derivative of R1 cells generating late-stage viable tetraploid embryos, and germ line-transmitting chimeras at a remarkable rate (21). GTR1 cells contain a transgene encoding the cardiomyocyte-specific α-myosin heavy chain promoter driving the puromycin-resistance gene and afford genetic selection of a virtually pure population of ES-derived cardiomyocytes (22). RNase protection analysis revealed that cell treatment with HBR following removal of LIF remarkably increased *GATA-4* and *Nkx-2.5* mRNA expression in both EBs and puromycin-selected cardiomyocytes (Fig. 2, *A* and *B*). These mRNAs encode, respectively, for a zinc finger-containing transcription factor and a homeodomain that have been shown to be essential for cardiogenesis in different animal species (29, 30), including humans (31). HBR also enhanced prodynorphin mRNA expression and the levels of dynorphin B, a natural κ opioid receptor agonist, in both EBs and ES-derived cardiomyocytes and in their incubation media (Fig. 2, *C* and *D*). This finding is particularly rewarding because we have recently shown that the prodynorphin gene and dynorphin B primed *GATA-4* and *Nkx-2.5* transcription (24) and triggered protein kinase C (PKC) signaling through complex subcellular redistribution patterning of targeted PKC isozymes, another major requirement for ES cell commitment to the cardiac lineage (22). Additionally, dynorphin B acted as an agonist of nuclear opioid receptors coupling nuclear PKC activation to the transcription of cardiogenic genes, indicating that intracrine signals for cardiac differentiation may also be fashioned by the prodynorphin gene and its related peptides (23).

Nuclear run-off transcription experiments on nuclei isolated from EBs or puromycin-selected cardiomyocytes revealed that the effects of HBR on *GATA-4*, *Nkx-2.5*, and prodynorphin mRNA expression occurred transcriptionally (Fig. 3). It is noteworthy that in both GTR1 and the parental R1 cells HBR failed to affect the transcription rate of *MyoD* and neurogenin-1, two genes involved in skeletal myogenesis and neuronal determination, respectively (Fig. 4). Indeed, the activation of a cardio-

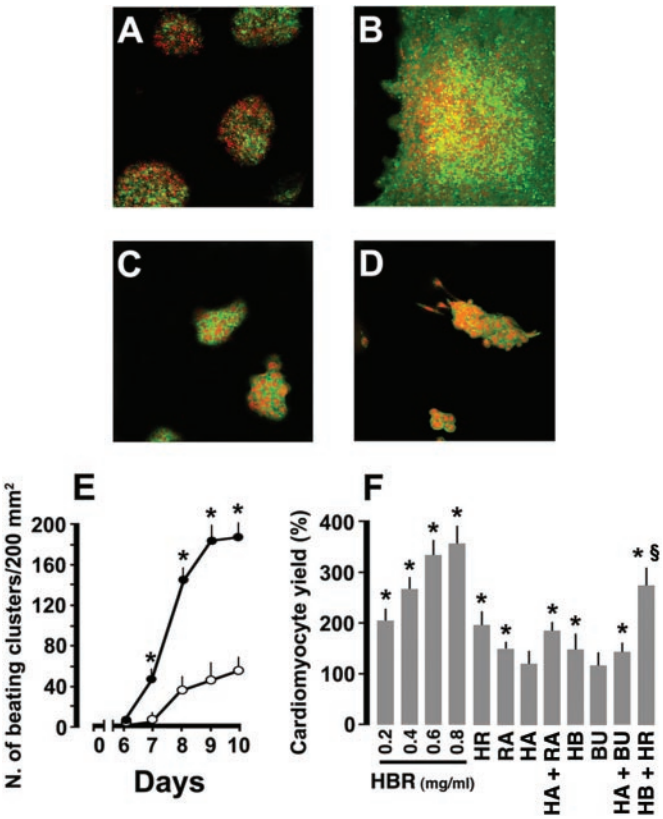

FIG. 5. **Effect of HBR and other hyaluronan esters on cardiogenic yield.** MHC immunostaining was assessed in cardiomyocytes derived from cells cultured without (*A*, *C*) or with (*B*, *D*) HBR as described in Fig. 2 (*A* and *B*, ×20 objective; *C* and *D*, ×40 visualization at low density plating). *E*, analysis of beating colonies in absence (○) or presence (●) of 0.75 mg/ml HBR. *F*, percentage changes in the number of beating colonies. From LIF removal throughout 4 days of puromycin selection, cells were exposed to increasing concentrations of HBR or treated in the presence of 0.75 mg/ml HR, 10^{-8} M all-*trans* retinoic acid (*RA*), 0.75 mg/ml hyaluronan (*HA*), 0.75 mg/ml HB, or 2.5 mM sodium butyrate (*BU*). These compounds were administered individually or according to the indicated combinations. In HB + HR, each monoester was administered at a concentration of 0.8 mg/ml. (Mean ± S.E.; $n = 6$). *, significantly different from untreated. §, significantly different from 0.8 mg/ml HBR.

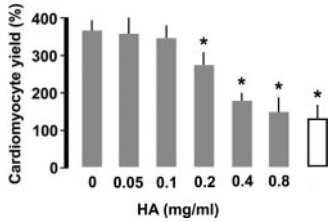

FIG. 6. **Hyaluronan counteracts the cardiogenic action of HBR.** From LIF removal throughout 4 days of puromycin selection, cells were exposed to 0.8 mg/ml HBR in the presence of the indicated concentrations of HA (*gray bars*). The *white bar* shows the effect of 0.8 mg/ml HA in the absence of HBR. Data (mean ± S.E.; $n = 6$) are expressed as percentage changes in the number of beating colonies (untreated controls = 100%). *, significantly different from HBR alone.

genic program of gene transcription was associated with an increase in the expression of the cardiac-specific genes *MHC* and myosin light chain-2V (Fig. 4). Treatment with 5 μM chelerythrine, a selective PKC inhibitor, following LIF removal and throughout 4 days of puromycin selection abrogated the transcriptional responses elicited by HBR (Fig. 3). Expression of the cardiac-specific transcripts *MHC* and myosin light chain-2V was concomitantly suppressed (Fig. 4). Similar results were yielded in the presence of 1 μM calphostin C, another PKC inhibitor (not shown). These findings are in keeping with the recruitment of PKC patterning in ES cell cardiogenesis (22, 23) and suggest that PKC-dependent mechanisms may also be involved in HBR action. Whether the differentiating effect of HBR may affect subcellular redistribution and/or gene expression profiling of targeted PKC isozymes is a subject for future investigations.

HBR did not affect the purity (percentage of MF 20-positive cells) of ES-derived cardiomyocytes (about 99% in untreated cells). MHC expression in cardiomyocytes from HBR-treated cells was further confirmed in immunofluorescence studies (Fig. 5, *A–D*). Interestingly, HBR exposure elicited a remarkable and dose-dependent increase in the number of spontaneously beating colonies as compared with untreated controls (Fig. 5, *E* and *F*). An ester of hyaluronan with RA alone (HR) induced a significantly lower increase in the number of beating colonies as compared with HBR (Fig. 5*F*). Treatment of EBs with low concentrations of RA between days 5 and 7 has been reported to increase cardiomyocyte differentiation (16). Although under these experimental conditions 10^{-8} (or 10^{-9} not shown), M all-*trans* RA significantly increased cardiomyocyte yield, the number of spontaneously beating colonies was considerably lower than that detected in HBR-exposed cells (Fig. 5*F*). Treatment with HA or BU alone only slightly increased the yield of puromycin-selected cardiomyocytes (Fig. 5*F*). A combined treatment of EBs with HA and RA or with HA and BU resulted in increased cardiomyocyte yield, as compared with the effect elicited by each individual compound, and mimicked the increase in the number of beating colonies observed in the presence of the corresponding hyaluronan monoesters, HR or HB (Fig. 5*F*). Akin to these observations, cell treatment with an association of HB and HR led to a further increase in the yield of ES-derived cardiomyocytes (Fig. 5*F*). On the whole, these findings indicate that each of the hyaluronan-grafted moieties in HBR may additively act in a cardiogenic response. Nevertheless, in the presence of HB plus HR the cardiomyocyte yield was still significantly lower than in HBR-treated cells. This observation suggests that a maximal differentiating response is achieved when both BU and RA are concomitantly internalized by the mixed ester. Probably, a competition for the same uptake system of the two monoesters altered an optimal intracellular BU/RA ratio and/or their timely sequence of action. The possibility that HBR uses an HA-related uptake system for cell entry is inferred from the results of competition experiments showing that HA dose-dependently counteracted the cardiogenic action of this glycoconjugate (Fig. 6). Whether an intracellular release of RA and BU may represent a major requirement for the biological activity of HBR or whether it may also act as a bioconjugate remains to be established.

Thus, HBR afforded the manipulation of a gene program of cardiogenic and cardio-specific genes, ultimately producing a high throughput of cardiogenesis from mouse ES cells. The finding that specification of a cardiogenic fate was attained without the need of gene transfer technologies makes HBR a novel potential tool for future investigations in ES cell-mediated cardiac rescue. Studies are in progress to shed additional light on the molecular events underlying the differentiating response primed by HBR in ES cells.

REFERENCES

1. Klug, M. G., Soonpaa, M. H., Koh, G. Y., and Field, L. J. (1996) *J. Clin. Investig.* **98,** 216–224
2. Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, V., Mickay, R., Nadal-Ginard, B., Bodine, D. M., Leri, A., and Anversa, P. (2001) *Nature* **410,** 701–705
3. Klug, M. G., Soonpaa, M. H., and Field, L. J. (1995) *Am. J. Physiol.* **268,** H1913-H1921
4. Wheatley, S. C., Isacke, C. M., and Crossley, P. H. (1993) *Development* **119,** 295–306
5. Camenisch, T. D., Spicer, A. P., Brehm-Gibson, T., Biesterfeldt, J., Augustine,

M. L., Calabro, A. Jr., Kubalak, S., Klewer, S. E., and McDonald, J. A. (2000) *J. Clin. Investig.* **106,** 349–360
6. Haegel, H., Dierich, A., and Ceredig, R. (1994) *Dev. Immunol.* **3,** 239–246
7. Tammi, R., Rilla, K., Pienimaki, J. P., MacCallum, D. K., Hogg, M., Luukkonen, M., Hascall, V. C., and Tammi, M. (2001) *J. Biol. Chem.* **276,** 35111–35122
8. Collis, L., Hall, C., Lange, L., Ziebell, M., Prestwich, R., and Turley, E. A. (1998) *FEBS Lett.* **440,** 444–449
9. Majumdar, M., Meenakshi, J., Goswami, S. K., and Datta, K. (2002) *Biochem. Biophys. Res. Commun.* **291,** 829–837
10. Zhang, S., Chang, M. C., Zylka, D., Turley, S., Harrison, R., and Turley, E. A. (1998) *J. Biol. Chem.* **273,** 11342–11348
11. Deb, T. B., and Datta, K. (1996) *J. Biol. Chem.* **271,** 2206–2212
12. Grammatikakis, N., Grammatikakis, A., Yoneda, M., Yu, Q., Banerjee, S. D., and Toole, B. P. (1995) *J. Biol. Chem.* **270,** 16198–16205
13. Sucov, H. M., Dyson, E., Gumeringer, C. L., Price, J., Chien, K. R., and Evans, R. M. (1994) *Genes Dev.* **8,** 1007–1018
14. Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dolle, P., and Chambon, P. (1994) *Cell* **78,** 987–1003
15. Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., and Mark, M. (1994) *Development* **120,** 2749–2771
16. Wobus, A. M., Kaomei, G., Shan, J., Wellner, M. C., Rohwedel, J., Ji, G., Fleischmann, B., Katus, H. A., Hescheler, J., and Franz, W. M. (1997) *J. Mol. Cell. Cardiol.* **29,** 1525–1539
17. Wolffe, A. P., and Pruss, D. (1996) *Cell* **84,** 817–819
18. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87,** 953–959
19. Dilworth, F. J., Fromental-Ramain, C., Yamamoto, K., and Chambon, P. (2000) *Mol. Cell* **6,** 1049–1058
20. McCue, P. A., Gubler, M. L., Sherman, M. I., and Cohen, B. N. (1984) *J. Cell Biol.* **98,** 602–608
21. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8424–8428
22. Ventura, C., Zinellu, E., Maninchedda, E., Fadda, M., and Maioli, M. (2003) *Circ. Res.* **92,** 617–622
23. Ventura, C., Zinellu, E., Maninchedda, E., and Maioli, M. (2003) *Circ. Res.* **92,** 623–629
24. Ventura, C., and Maioli, M. (2000) *Circ. Res.* **87,** 189–194
25. Coradini, D., Pellizzaro, C., Miglierini, G., Daidone, M. G., and Perbellini, A. (1999) *Int. J. Cancer* **81,** 411–416
26. Benedetti, L. M., Joshi, H. N., Goei, L., Hunt, J. A., Callegaro, L., Stella, V. J., and Topp, E. M. (1991) *New Polymeric Mater.* **3,** 41–48
27. Höfle, G., Steglich, W., and Vorbrüggen, H. (1978) *Angew. Chem. Int. Ed. Engl.* **17,** 569–583
28. Maryanoff, C. (May 10, 1988) U. S. Patent 4743400
29. Biben, C., and Harvey, R. P. (1997) *Genes Dev.* **11,** 1357–1369
30. Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., and Harvey, R. P. (1993) *Development* **119,** 419–431
31. Benson, D. W., Silberbach, G. M., Kavanaugh-McHugh, A., Cottrill, C., Zhang, Y., Riggs, S., Smalls, O., Johnson, M. C., Watson, M. S., Seidman, J. G., Seidman, C. E., Plowden, J., and Kugler, J. D. (1999) *J. Clin. Investig.* **104,** 1567–1573