Keywords: anticoagulant, Chinese hamster ovary cells, heparin, LC-MS, metabolic engineering, transcriptional regulation, translational regulation

Submitted: 05/18/12
Revised: 05/24/12
Accepted: 05/28/12

http://dx.doi.org/10.4161/bioe.20902
*Correspondence to: Susan T. Sharfstein; Email: ssharfstein@albany.edu

**Addendum to:** Baik JY, Guerini L, Yang B, Dahta P, Zhang T, Glass CA, et al. Metabolic engineering of Chinese hamster ovary cells: Towards a bioengineered heparin. Metab Eng 2012; 14:81-90. PMID: 22326251; http://dx.doi.org/10.1016/j.ymben.2012.01.008.

Heparin is the most widely used pharmaceutical to control blood coagulation in modern medicine. A health crisis that took place in 2008 led to a demand for production of heparin from non-animal sources. Since Chinese hamster ovary (CHO) cells are capable of producing heparan sulfate (HS), a related polysaccharide naturally, and heparin and HS share the same biosynthetic pathway, we hypothesized that heparin could be produced in CHO cells by metabolic engineering. We developed stable human N-deacetylase/N-sulfotransferase (NDST2) and mouse heparan sulfate 3-O-sulfotransferase 1 (Hs3st1) expressing cell lines based on the expression of endogenous enzymes in the HS/heparin pathways of CHO-S cells. Both activity assay and disaccharide analysis showed that engineered HS attained heparin-like characteristics but not identical to pharmaceutical heparin, suggesting that further balancing the expression of transgenes with the expression levels of endogenous enzymes involved in HS/heparin biosynthesis might be necessary.

Heparin is a highly sulfated, glycosaminoglycan (GAG) that consists of repeated disaccharides, L-iduronic acid (IdoA) or D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) with or without sulfogroups (Fig. 1). Heparin is widely used clinically as an anticoagulant, particularly for surgery and kidney dialysis.1 Greater than 100 tons of heparin are used annually, with a market value of ~$7 billion.9 Virtually all clinically available heparin is animal-derived, primarily from porcine intestinal mucosa. It is estimated that only three doses of heparin can be obtained from one animal.3 In early 2008, there was a marked increase in serious adverse events associated with heparin therapy, with nearly 100 associated deaths in the United States alone. After thorough investigation, it was discovered that the heparin injected into patients had been contaminated by over-sulfated GAGs,4 highlighting the potential risks of contamination from the current methods of heparin production, extraction from animal tissues. While chemoenzymatic approaches to developing a bioengineered heparin have been reported,5-8 the yields are still quite low, creating an impetus for other approaches to create a bioengineered heparin.

Chinese hamster ovary (CHO) cells, the most widely used cells for therapeutic protein production, are good candidates for production of a bioengineered heparin. They express many of the enzymes involved in glycosylation; they are relatively safe from biological contamination such as viruses, and they can be adapted to suspension culture and easily scaled up. More importantly, CHO cells produce heparan sulfate (HS), a less sulfated GAG that has some basic disaccharide units as heparin. HS also has considerably lower anticoagulant activity than heparin due to lacking a unique pentasaccharide motif, an anti-thrombin (ATIII) binding site (Fig. 2).9-11 Similarities and differences between HS and heparin are summarized in Table 1.
the chains resulting in O-sulfo poor and GlcNAc, GlcA-rich chains. Since heparin and HS share a common biosynthetic pathway, we hypothesized that CHO cells could be metabolically engineered to produce heparin. In a recent work, we evaluated the expression levels of metabolic enzymes and isozymes involved in the biosynthetic pathway of HS/heparin and identified missing functionalities in CHO cells. We then constructed stable cell lines that expressed the relevant enzymes by genetic engineering. Finally, the structure and activity of the engineered HS was characterized.

Metabolic Engineering Strategies

We used rat mast cells, natural producers of heparin, to identify the required expression of HS/heparin biosynthetic enzymes and compared it to that of CHO-S cells (Invitrogen). Based on the RT-PCR and western blotting results, we determined that CHO-S cells did not express Ndst2 and showed minimal expression of Hs3st1, which are known to be critical for anticoagulant heparin biosynthesis (Fig. 3). Ndst2 plays an important role in the introduction of N-sulfo groups into GlcNAc, which in turn, is important for subsequent sulfonation of the growing HS chain. Absence of Ndst2 expression can explain the low level of N-sulfo groups in CHO-S HS. Hs3st1 is responsible for the formation of the unique ATIII binding pentasaccharide, which makes heparin an important anticoagulant therapeutic molecule. A recent study of CHO-K1 genome and transcriptome sequences verified that these enzymes were not expressed in CHO cells. Therefore, Ndst2 and Hs3st1 were engineered into the CHO-S cells. Two stable cell lines (Dual-3 and Dual-29) were selected and HS from these cells and cultured media was isolated for further characterization.

Extensive studies by Lindahl and coworkers have shown that heparin and HS are biosynthesized through a similar pathway. After formation of a common tetrasaccharide (xylose-galactose-galactose-glucuronic acid),12 the chains are polymerized by the sequential addition of basic disaccharides (GlcA-GlcNAc) followed by a series of modifications including N-deacetylation and N-sulfonation of GlcNAc residues, epimerization of GlcA to IdoA, and finally, the introduction of O-sulfo groups at different positions of the glucosamine (GlcN) and uronic acid residues (Fig. 3).13 Complete or nearly complete modification of this nascent GAG chain results in a highly N-sulfo, O-sulfo, IdoA-rich GAG commonly referred to as heparin, which serves as the source of material for further fractionation to generate pharmaceutical heparin. HS is characterized by partial modification of the chains resulting in O-sulfo poor and GlcNAc, GlcA-rich chains. Since heparin and HS share a common biosynthetic pathway, we hypothesized that CHO cells could be metabolically engineered to produce heparin. In a recent work, we evaluated the expression levels of metabolic enzymes and isozymes involved in the biosynthetic pathway of HS/heparin and identified missing functionalities in CHO cells.16 We then constructed stable cell lines that expressed the relevant enzymes by genetic engineering. Finally, the structure and activity of the engineered HS was characterized.
Characterization of Engineered HS

The engineered HS was isolated from cell pellets and analyzed by an anti-factor Xa anticoagulation assay to determine whether the engineered HS showed increased anticoagulant activity. Since cell-membrane-bound HS proteoglycans can be shed through the action of proteases,19,20 the engineered HS was also purified from the culture medium and analyzed by the anti-factor Xa assay. As shown in Figure 4, the HS extracted from Dual-3 and Dual-29 cell pellets shows significantly increased anticoagulation activity (7.5-fold and 6.8-fold, respectively) compared with the HS from CHO-S host cells. Unfortunately, the anticoagulant activity of the dual-expressing cells is still considerably lower than that of the pharmaceutical heparin. However, the pharmaceutical heparin has been fractionated to obtain high anticoagulant activity, whereas the bioengineered HS used in the activity assay was unfractionated, which may explain some of the difference in activity. The HS purified from culture media of Dual-3 and Dual-29 also shows increased anticoagulation activity (52.9-fold and 97.2-fold, respectively) compared with CHO-S cells.

The total GAGs were isolated from culture media and analyzed by reverse-phase ion-pairing ultra-performance liquid chromatography mass spectrometry (RPIP-UPLC-MS).21 The amount of total HS/heparin and ratio of disaccharide components are summarized in Table 2.
...and Figure 5. The amount of HS/heparin secreted by the engineered cells was increased nearly 10-fold in comparison with the wild-type (a smaller increase was observed in the cell-associated HS/heparin). A substantial increase in sulfation was also observed, primarily N-sulfation, confirming the activity of NDST2. While this represents a significant improvement, it is still quite different from the trisulfated species that characterize the pharmaceutical heparin. Therefore, an engineered HS although tri-sulfated disaccharides while the major species in dual-expressing cell lines are NS disaccharides while the major species in heparin are tri-sulfated disaccharides, suggesting that the expression level of NDST2 is too high, which overwhelms the actions of other enzymes. The mechanism that controls the HS/heparin biosynthetic pathway is also still unclear, but one widely supported hypothesis is that NDST is involved in the termination of sulfonation in HS/heparin. Therefore, overexpression of NDST might terminate the sulfonation of HS/heparin before other sulfotransferases were able to act on the HS chain, which means that it may be necessary to balance the expression levels of the transgenes with the endogenous enzymes. Our current study is focusing on balancing the metabolic engineering to produce an engineered HS identical to the pharmaceutical heparin in terms of structure and activity. These requiring understanding the activity of the HS/heparin biosynthetic enzymes in the Golgi apparatus. Assays to establish the activity of GAG biosynthetic enzymes are under development in our laboratory.

Fine tuning of enzyme expression will also be necessary to precisely control the metabolic flux; this may be performed by either transcriptional or translational regulation of enzyme expression. In E. coli or S. cerevisiae, the expression levels of enzymes involved in metabolic pathways have been controlled by promoter engineering, gene titration or gene circuit engineering. In mammalian systems, the control and tuning of expression levels has been less widely practiced. This is likely due to a lack of genetic tools—common expression vectors for mammalian cells still utilize genetic elements [e.g., the human cytomegalovirus (CMV) promoter] that generate the highest levels of recombinant gene expression. Yet depending on the gene, product of interest, or desired cell phenotype, a high level of expression generated by an exogenous vector may prove to be suboptimal, physiologically irrelevant or even toxic. One approach to controlling expression levels is to use an inducible system. For example, with tetracycline-inducible systems, tetracycline or its analogs can be added to activate or deactivate synthetic transcription factors in a dose-dependent and tunable manner. There are two potential drawbacks to using inducible systems: (1) turning an inducible system “off” generally leaves a level of leaky expression, which can still be higher than the optimized level one seeks; (2) optimization of multiple genes (where expression of one gene is independent of the other) requires multiple orthogonal inducible systems. A constitutive system that allows for expression level control can potentially avoid both of these drawbacks. Recently, Ferreira et al. developed a constitutive promoter library that is well suited for the tuning and optimization of genes exogenously expressed in mammalian cells. They performed site-directed mutagenesis at the CAAT and TATA box transcription factor binding sites and degenerate PCR mutagenesis to generate a total of 25 mutant CMV and EF1α promoters capable of an approximately 40-fold range in transcription levels. Furthermore, using a library of vectors employing these promoters, Ferreira et al. evaluated a broad range of oncogenic Ras expression levels. They were able to determine that the contribution of oncogenic Ras to imatinib drug resistance has an optimized, nonlinear behavior. A pre-B leukemia cell line...
proliferated optimally in the presence of insulin. When rho GDP was increased above 0.3, HS/heparin level was approximately equivalent to the endogenous wild-type Ras expression level. Higher expression levels subsequently led to decreased proliferation rates. We anticipate that using a library of constitutive promoters to simultaneously optimize the expression levels of both HS/heparin and EXT will increase both the yield and activity of recombinant HS.

Maximizing Total Expression of HS/Heparin

In addition to the challenges involved in obtaining HS/heparin with the correct structure for biological activity, maximizing the overall expression of heparin will also be necessary. In the dual-expressing cell lines, the amounts of HS/heparin in the media were increased significantly compared with HS/heparin extracted from the cells (4.5-fold to 9-fold), which implies that HS/heparin on the membrane are shed into the medium. Hence, we appear to have increased the metabolic flux through this pathway by metabolic engineering. Increased HS/heparin in the media also supports the hypothesis that bioengineered HS chains will still use the HS core proteins, syndecan and glypican, for extracellular targeting. Extracellular secretion using these HS core proteins will greatly simplify heparin purification as it will eliminate the necessity of cell lysis to recover the heparin. However, tuning genetic control through promoter engineering will lead to increased HS synthesis as has been observed in human embryonic kidney cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was funded by a grant from the National Institutes of Health (RO1GM090127).

References

1. Bigelow WG. Mucinous Heparin: The Key to Open Surgery. Toronto-Montreal: McGraw-Hill Ryerson 1990.
2. Lin H, Zhang, Z, Lishtew KJ, Levens learned from the combination of heparin. Nat Prod Rep 2009; 26:333-339; PMID:19264956; http://dx.doi.org/10.1039/b804986a.
3. Lishtew KJ, Gentry NS, Production and chemical processing of low molecular weight heparin, Semin Thromb Hemost 2012; 38:144-152; PMID:22580370; http://dx.doi.org/10.1055/s-0032-130166.
4. Gareux BC, Bocavi D, Shriver Z, Nagy A, Vassanadeh K, Boire A, et al. Overexpression of heparin sulfate in a contaminant in heparin concentrate with adverse clinical events. Nat Biotechnol 2010; 28:1318-1321; PMID:20906747; http://dx.doi.org/10.1038/nbt1407.
5. Lin R, Xu Y, Chen M, Warren M, Zhou X, Bridges AT, et al. Chemoenzymatic design of heparin sulfate oligosaccharides. J Biol Chem 2010; 285:34240-34250; PMID:20865501; http://dx.doi.org/10.1074/jbc.M110.110601.
6. Xu Y, Minou S, Takaldini M, Xu H, Liu B, Jong J, et al. Chemoenzymatic synthesis of heparin sulfate oligosaccharides. J Biol Chem 2010; 285:34244-34250; PMID:20865502; http://dx.doi.org/10.1074/jbc.M110.110602.
7. Wang Z, Yang B, Zhang Z, Ly M, Takaldini M, Minou S, et al. Control of the heparin sulfate N-acylation leads to an improved biografted heparin. Appl Microbiol Biotechnol 2011; 90:959-968; PMID:21325501; http://dx.doi.org/10.1007/s00253-011-2950-0.
8. Marston J, Rosenberg RD. Anticausal activity of heparin-sulfate oligosaccharide and the vascular endothelium. J Am Chem Soc 1997; 119:446-456; PMID:2389020; http://dx.doi.org/10.1021/ja962118d.
9. Capilla I, Lishtew B. Heparin-protein interaction. Angew Chem Int Ed Engl 2005; 44:109-122; PMID:16091530; http://dx.doi.org/10.1002/anie.200401377; 2005; 44:109-122; PMID:16091530; http://dx.doi.org/10.1002/anie.200401377.
10. Munnuk L, Benczyk Z, Kovacs B, Kondwandt J. Antibody deficiency and its laboratory diagnosis. Clin Chem Lab Med 2010; 48:65-78; PMID:20163218; http://dx.doi.org/10.1515/cclm.2010.368.
11. Roberton MC, Hemes AH, Hark M, Ogden S, Linkal U. A proteoglycan form of heparin and its oligosaccharides in single-chain molecule. J Biol Chem 1978; 253:4007-4013; PMID:608203.
12. Uda M, Ishikawa M. Okita use of an assembly of ligand binding sites in heparin sulfates. Ann Rev Biochem 2006; 75:497-517; PMID:16285500; http://dx.doi.org/10.1146/annurev.biochem.75.1.497.
13. Coss B. Structure and biological activity of heparin. Adv Cardiol Clin Pract 1995; 45:10-16; PMID:7498029; http://dx.doi.org/10.1055/s-0032-130166.
14. Varan AV, Kannabhiran T, Tirodkar H, Xie J, Harris K, Dillon S, et al. Glycosylation of proteoglycan heparin in marine embryonic stem cell differentiation. J Proteome Res 2007; 6:1874-1881; PMID:17591847; http://dx.doi.org/10.1021/pr0607046.
15. Bai JY, Gasimli L, Yang B, Datta P, Zhang F, Linhardt RJ. The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. Nat Biotechnol 2009; 27:740-744; PMID:19239216; http://dx.doi.org/10.1038/nbt1800.
16. Linhardt RJ. Heparin-protein interactions. Annu Rev Biochem 2002; 71:435-471; PMID:12045103; http://dx.doi.org/10.1146/annurev.biochem.71.110601.135458.
17. Sugahara K, Tsurugai H. Heparin and heparin-like compounds in biology. Crit Rev Biocem Mol Biol 2006; 31:67-94; http://dx.doi.org/10.1080/104092306008945292.
18. Xu J, Nguyen H, Liao H, Xie Z, Lin X, et al. The genomic sequence of the Chinese hamster ovary (CHO-K1) cell line. Nat Biotechnol 2001; 19:75-81; PMID:11189602; http://dx.doi.org/10.1038/8432.
19. Lunde MD, Guevara M, Beccati D, Shriver Z, Naggi A, Tani A, et al. Chemoenzymatic design of heparan sulfate oligosaccharides. Metab Eng 2007; 9:193-207; PMID:17239639; http://dx.doi.org/10.1016/j.ymben.2006.11.002.
20. Alpipe H, Bucche C, Novogor E, Stoupakova G. Tuning genetic control through proteome engineering. Dyn. Nat Acad Sci USA 2005; 102:12832-12837; PMID:16123130; http://dx.doi.org/10.1073/pnas.0504661102.
21. Michelot-Peterson K, Isaila M, Engineering prokaryotic gene circuits. FEMS Microbiol Rev 2010; 34:517-571; PMID:20608181; http://dx.doi.org/10.1111/j.1574-6976.2009.00194.x.
22. Ferris FS, Sullivan RA, Wang CL. Terminator-Regulated Expression. Implemented through Transcriptional Activation Combined with Promoter and Dual Repression ACS Synthetic Biology 2012; 1:154-166; http://dx.doi.org/10.1021/as200074v.
23. Ferris JS, Laskarz EB, Wang CL. Modulating specific gene expression levels by using neutral vectors equipped with synthetic promoters. Syn Synth Biol 2012; 3:119-138; http://dx.doi.org/10.1016/j.yssb.2012.01.009.
24. Ferris JS, Laskarz EB, Wang CL. Quantitative assessment of promoter overexpression via shRNA delivery of repressor Antibiotic Res Comb Chem 2012; 4:108-104; PMID:22810882; http://dx.doi.org/10.1016/j.yssb.2012.01.009.
25. Belloni C, Donati R, Gensini G, Neumann BJ, Bresolin N, et al. Contribution of EXT1 and EXT2 to heparin chain polymerization, will lead to increased HS synthesis as has been observed in human embryonic kidney cells.

www.landesbioscience.com

Bioengineered

©2012 Landes Bioscience. Do not distribute.