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European Community and US-FDA Approval of Recombinant Human Antithrombin Produced in Genetically Altered Goats

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Thrombin and factor Xa play a central role in thrombogenesis in both medical and surgical patients. Antithrombin (AT) is the key inhibitor, which controls the action of these enzymes in hypercoagulable states. The AT concentrates prepared from human blood have been used to treat patients with thrombotic disorders and heparin resistance. The AT concentrates are prepared from pooled human plasma and beside limited supply, suffer from viral and other biological contaminants. The availability of recombinant human AT (rhAT) obtained from genetically engineered goats provide a biologically equivalent product that can be used in practically all indications where human AT is indicated including heparin resistance. Moreover, because of its high affinity to heparin and related drugs, recombinant AT can also be developed in further indications. On review of the preclinical and clinical data on the safety and efficacy, the European Union and U.S. Food and Drug Administration (US-FDA) have recently approved the use of rhAT in specified clinical indications.

Keywords: recombinant antithrombin; hypercoagulability; thrombosis; genetically altered goats; antithrombin deficiency

Introduction

Recombinant technology has contributed significantly to the development of proteins for the treatment of thrombotic disorders. Products, such as recombinant hirudin, thrombomodulin, tissue factor pathway inhibitor (TFPI), and its molecular variants have been developed using conventional methods. More recently, transgenic animals have also been used in the production of recombinant proteins.

Nowadays recombinant human antithrombin (rhAT) is a product of DNA technology which uses transgenic genetically engineered goats. In this procedure DNA sequence of human AT has been introducted to a memory gland that regulates a specific DNA sequence. The goat-derived product is similar to human plasma-derived AT with the exception of posttranslational glycosylation profile.[1] Although this product has higher heparin affinity and shorter half-life, its behavior in the in vitro assays is similar to human plasma-derived AT.[2] Extensive preclinical and clinical studies have been carried out for safety and efficacy on the recombinant human AT.

In July 2006, based on the information submitted, the European Union approved the rhAT

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(ATryn), for the prophylaxis of venous thromboembolism in surgical patients with congenital AT deficiency.[3] On February 6, 2009, the US Food and Drug Administration (FDA), Blood Product Advisory Committee (BPAC) approved ATryn for the treatment of patients with hereditary AT deficiency undergoing surgical or obstetrical procedures.[4]

Antithrombin and its Role in the Regulation of Hemostasis

Antithrombin is a 58-kd glycoprotein belonging to a group of inhibitory protein factors known as serpins (serine protease inhibitors).[5] It plays an important role in the regulation of coagulation by the inhibition of thrombin (factor IIa), factor Xa, and to a lesser extent factors IXa, XIa, and XIIa. Approximately 80% of the endogenous inhibitory effects against thrombin are provided by AT. Antithrombin produced in the hepatic parenchyma has a circulating concentration of 122 to 140 g/mL, which is equivalent to an AT activity of 100 IU/dL with a half-life of 2 to 3 days.[6] The mechanism of action of AT involves the interaction of its active arginine site with the active serine residue on thrombin. The AT activity is dependent on its reactive site, which performs the proteolytic function, and the heparin-binding site. Heparin augments the activity of AT about thousandfold by binding to a lysine residue and causing a conformational change at the arginine reactive center.[7-9] Antithrombin causes antiinflammatory effects by the inhibition of the coagulation process and its interaction with the endothelium.[10]

The cleaved and latent forms of AT are known to be the potent inhibitors of angiogenesis and tumor growth in animal models.[11] The crucial role of AT in the regulation of coagulation may be ascertained by the increased incidence of thrombotic complications seen in patients with inherited or acquired AT deficiency.[12] Antithrombin deficiency is generally diagnosed in patient with recurrent venous thrombosis and pulmonary embolism.

Inherited AT Deficiency

In the normal population, the incidence of inherited AT deficiency has been estimated to be between 1:2000 and 1:5000.[13,14] Based on the functional and immunochemical AT analyses, a classification of AT designated as either type I or type II was proposed.[15] Antithrombin levels of at least 70% are required for optimum and effective inhibition of the serine proteases involved in the coagulation processes.[16] Functional AT levels are generally reduced to below 50% of normal due to AT deficiency of types I and II.[17]

Acquired AT Deficiency

Acquired AT deficiency may result from liver dysfunction (coagulopathy), malnutrition, asparaginase therapy, heparin therapy, sepsis/disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, premature birth, nephritic syndrome, or major surgical procedures, such as cardiopulmonary bypass (CPB) procedures.[18] Although the levels at which AT deficiency manifests thrombotic complications is unclear and may depend on the cause of deficiency, levels <50% to 60% in the presence of sepsis are generally associated with poor outcome. Patients with AT <20% levels usually have a fatal outcome.[19]

Therapeutic Use of AT

Antithrombin concentrates may be given exogenously for thromboprophylaxis of various surgical and medical indications. Antithrombin replacement, only available as a pooled plasma-derived product, has an uncertain risk of transfusion-transmitted infection despite effective viral inactivation. Recombinant AT (ATryn), approved earlier in Europe and recently in the United States, made from goat milk genetically modified to provide a useful alternative treatment option for all indications where human plasma-derived AT is used.

The genetic engineering of livestock, first suggested in 1982, has been a topic of much discussion to yield improved animals for the dairy and ranching industries.[20-23] Most applications of transgenic technology in dairy animals have been with the intent of recovering the desired protein from the milk for other uses; for example, to produce pharmaceuticals[24] and nutraceuticals[25] from the mammary glands. Goats and cows are the most commonly used animals.

Antithrombin Concentrates

The intravenous administration of AT concentrates may be given to patients with AT deficiency

especially during surgery or labor when they are at increased risk of thromboembolism. Antithrombin concentrates may also be given in situations where thromboprophylaxis with anticoagulant drug is not possible, because of an increased risk of bleeding (neurosurgery). The criteria as to which individuals with AT deficiency should be treated and the ones which should not are not fully established. Antithrombin can be replaced in deficient patients using an intravenous infusion of a highly purified, human plasma-derived AT protein (pdAT; Thrombate III; Talecris Biopharmaceutics NC, USA) can be administered to replace AT in deficient patients. Several other products are available in other countries. After screening for hepatitis, HIV, and other viruses, such as parvovirus B-19, concentrates are prepared from the blood of tens of thousands of donors. The plasma is then purified, resulting in an AT concentrate. Heat inactivation or special filtration techniques are used to inactivate any potential virus contaminants. The US-FDA has approved the use of plasma-derived AT for use in patients with hereditary AT deficiency.[4] In patients with a congenital deficiency of AT, replacement/prophylaxis is recommended before or following major surgery, during bed rest longer than 24 hours (because of increased risk of thrombosis), for thrombosis during pregnancy to allow heparin to be effective, and for acute deep vein thrombosis (DVT)/pulmonary embolism.

Many acquired causes have been associated with AT deficiency. In patients with shock and DIC due to trauma, sepsis, or hepatic coma, the duration of DIC symptoms was significantly shorter in patients administered AT alone than in patients administered heparin alone. However, there was a greater preponderance of bleeding manifestations in patients with DIC due to polytrauma, who were administered heparin.[26]

Recombinant AT

Recombinant human AT, a transgenic form manufactured by GTC Biotherapeutics (formerly Genzyme Transgenics Corporation, Boston, MA) has undergone extensive clinical trials in thrombotic disorders. This rhAT is produced by inserting human DNA into the goat cells and the targeted protein is excreted in the female offspring's milk. The transgenic goats have been cloned in collaboration with the Louisiana State University Agriculture Center. The rhAT is considered safer and more cost-effective than the currently available plasma-derived product. Currently, rhAT is also being investigated in cancer and acute lung injury.[27]

ATryn (branded form of GTC's rhTA) is by far the first medicine produced using genetically engineered animals.[2] It is manufactured by the US company GTC Biotherapeutics (Framingham, MA) from goats milk that have been genetically altered to produce human AT, a plasma protein with anticoagulant properties. Using the microinjection technique, human AT genes were inserted into the cell nucleus of their embryos.[2] It is expected that a genetically modified goat can produce the same amount of AT in a year as 90 000 blood donations.[2] Goats were chosen for the process because of their more rapid reproductive cycles than cattle and for the production of more protein than rabbits or mice.

On February 6, 2009, ATryn was approved by the FDA for the treatment of patients with hereditary AT deficiency undergoing surgical or childbirth procedures.[4] The Center for Veterinary Medicine of the FDA also approved the genetic makeup of the goats used to manufacture ATryn. The Ovation Pharmaceutical Company, Deerfiel, IL has the sole rights to sell ATryn in the United States. In 2009, ATryn is expected to be available in the US market.[28]

Production of the Transgenic Animal

The transgenic goat was obtained after microinjection of the DNA construct (transgene), comprising the cDNA of human AT and the regulatory elements (promoter) of the goat β-casein gene into the pronucleus of a goat embryo. This transgene acting as a promoter drives the expression of rhAT in the mammary gland. The selection of the first transgenic goat (male founder) was based on the DNA sequence integrity, ability of germ line transmission of a transgene, and the level of rhAT in the milk.[2,29,30] Goat breeding was reportedly accomplished through a combination of natural breeding and artificial insemination. The genetic stability of the transgene has been demonstrated to last up to 7 generations of goats. The additional steps are undertaken to control the genetic stability of production goats. Factors considered for the evaluation of goats to the production herd included genotyping, assessment of milk composition and content of rhAT, microbiological and adventitious agent screening of the milk, and general examination of animal health.

The milk collected from the genetically engineered goats is frozen and then shipped to the facility for purification and further processing. A milk-pooling strategy has been incorporated to optimize the consistency of the source material, which shows certain variability with regard to the milk composition and glycosylation profile of the rhAT molecule, depending on the production animal or lactation timing. The composition of the milk pool is tested before it enters the purification process.

Purification of ATryn Drug Substance (DS)

The purification process starts with thawing, pooling, and clarification of the source material, followed by filtration steps, passage through 3 chromatography columns, and filtration through a virus retention nano filter.[2] The DS is formulated prior to the final production steps. Removal of milk-derived impurities by the purification process was adequately validated by the analyses of milk production pool steps and the DS batches derived from the validation studies or designated for commercial distribution.[30] Validated analytical assays were reported to be used to demonstrate the efficient removal of milk-related impurities. Furthermore, the safety of the impurities profile of ATryn has been confirmed by nonclinical and clinical studies.[30,31]

Comparison of rhAT and pdAT

Recombinant human AT, a glycoprotein comprising 432 amino acids, with a molecular weight of approximately 57 215 d is known to have a structure consistent with published reports on pdAT with 4 methionine residues identified to serve as data on pdAT. Recombinant human AT when compared to pdAT, based on the glycosylation profiles and affinity to heparin, showed significant differences. Basically, the glycosylation profile of ATα differs from pdAT in terms of monosaccharide composition, oligosaccharide population, as well as glycoform species. These differences result in 4-fold increased affinity to heparin and shorter half-life of rhAT compared to pdAT.[2] Differences were also noted based on heparin-binding affinity analysis in that the plasma-derived pdAT was composed of ~90% α isoform (low-binding affinity to heparin) and ~10% β isoform (high-binding affinity species).[2] The ATα demonstrates a pdAT "β-like" affinity without any detectable impact on the in vitro bioassay performed in excess of heparin. The functional in vitro assays for rhAT activity, such as inhibition of thrombin and factor Xa are unaffected because they are performed in the presence of excess amounts of heparin.[2]

Clinical Trials

A phase III, double-blind, placebo-controlled, multicenter study was conducted to evaluate the efficacy of rhAT to restore heparin responsiveness in heparin-resistant patients scheduled to undergo cardiac surgery necessitating CPB.[32] In this study, 54 heparin-resistant patients were randomized into 2 groups. One group received 75 U/kg rhAT, while the other group received normal saline. Heparin resistance was diagnosed when the activated clotting time (ACT) was less than 480 seconds after 400 U/kg heparin. The treatment was considered a failure in the treatment group, if the ACT remained for less than 480 seconds and patients were transfused with 2 units of fresh frozen plasma. The results showed that 19% of the patients on rhAT received fresh frozen plasma when compared to 81% of patients in the placebo group ($P < .001$). During the hospitalization period, 48% of the patients on rhAT received fresh frozen plasma compared to 85% on placebo ($P = .09$). Thus, in this study, placebo patients required higher doses of heparin ($P < .005$). There were no serious effects due to rhAT that were reported. However, there was a report of increased blood loss 12 hours postoperatively in the rhAT group, continuing upto 24 hours. This study concluded that the treatment with 75 U/kg rhAT was effective in restoring heparin responsiveness and promoting therapeutic anticoagulation in heparin-resistant patients, thereby decreasing the requirement of heparin and fresh frozen plasma.

A similar study reported comparatively favorable results in patients who received rhAT (n = 28) versus placebo (n = 24) for the management of human resources (HR) during cardiac surgery requiring CPB.[33] In this study, rhAT restored heparinization responses and decreased activation of coagulation in CPB patients resistant to this anticoagulant.

Five patients with hereditary AT deficiency, who underwent 6 surgical procedures were treated perioperatively.[34] Dosing was at the discretion of the investigators, with a goal of maintaining an AT activity of 80% to 150%. There was no clinical evidence of

thrombosis or bleeding. Of the 5 patients, 4 had postoperative duplex ultrasound studies of the lower extremities, which showed no evidence of acute thrombosis. Four patients were tested for antirhAT antibodies postoperatively and were found to be negative.

A multicentric trial evaluated the efficacy and safety of prophylactic intravenous administration of ATαto hereditary AT-deficient patients in high-risk situations, including elective surgery, childbirth, or cesarean section.[35] Antithrombin αwas administered prior to and during the high-risk period for restoration and maintenance of AT activity at 100% normal. Heparin, low-molecular-weight heparin, and/or vitamin K antagonists were used according to the standard of care. Safety was assessed based on adverse events (AEs) and laboratory evaluations. For a mean period of 7 days, 5 surgical and 9 obstetrical hereditary AT deficiency patients received ATα. No clinically overt DVT was found. Ultrasonograms identified signs of acute DVT in 2 of 13 evaluable patients. No ATα-related AEs were reported. No patient developed anti-ATαantibodies. This study suggests that ATαis a safe and effective alternative to human plasma-derived AT for treating hereditary AT deficiency in patients at high risk of thromboembolic events.

These clinical trials clearly suggest that rhAT produced its effects in a similar manner as those expected with pdAT and therefore these 2 products are comparable.

Advantages

The clinical use of pasteurized AT concentrates has been limited by availability and concerns regarding the risk of infectious agent transmission by pooled products that involve large donor exposures. Although the AT concentrate is used in coronary artery bypass grafting procedures, it has not been approved for this indication and for other causes of acquired AT deficiency in the United States. The development of transgenic, recombinant technology has facilitated the production of considerable quantities of pure proteins for potential use in humans. Some of the potential advantages of transgenically produced recombinant proteins include unlimited supply and a negligible risk of infectious agent transmission compared to plasma-derived proteins.

The need for an alternative source of AT is evidenced by the fact that pdAT has not always been readily available.[34] Moreover, restrictions on donor selection and the increased need for other plasma-derived proteins, such as coagulation factors, and immunoglobulins may further risk pdAT availability in the future. Despite the use of modern virus removal and inactivation methods in the production of pdAT, there is still a potential risk of transmission of blood-borne infectious agents.[36,37]

Disadvantages

One study reported that there was no development of antibodies with the use of rhAT.[35] However, the data on repeated exposure with this product are limited. Immunogenicity can be evaluated in patients with repeated exposure to ATryn through an immunosurveillance program. ATryn is contraindicated in patients with known hypersensitivity to goat milk and goat milk proteins. Because of the higher affinity to heparin and related agents, rhAT may exhibit hemorrhagic effects if the dosage is not optimized. Moreover, caution must be exercised when rhAT is administered concomitantly to patients treated with heparin and related drugs.

Indication and Usage

ATryn is a recombinant AT indicated for the prevention of perioperative and peripartum thromboembolic events in hereditary AT-deficient patients.[4] It is not indicated for treatment of thromboembolic events in hereditary AT-deficient patients.

The dosage of ATryn is to be individualized based on the patient's pretreatment functional AT activity level (expressed in % of normal) and body weight (expressed in kg) and using therapeutic drug monitoring. The goal of the treatment is to restore and maintain functional AT activity levels between 80% and 120% normal (0.8–1.2 IU/mL).

Safety

ATryn has an acceptable safety profile when used for prevention of perioperative and peripartum thromboembolic events in hereditary AT-deficient patients at the recommended dose schedule. The serious adverse reaction that has been reported in clinical studies is hemorrhage (intraabdominal, hemarthrosis, and postprocedural). The most common AEs reported in clinical trials at a frequency of $\geq$5% are

hemorrhage and infusion site reaction.[4] Concomitant administration of heparin, low-molecular-weight heparins, and related products such as fondaparinux may enhance the pharmacodynamic action of ATryn. Safety with repeated exposure should be evaluated in a postmarketing study.

Conclusions

Recombinant human AT derived from genetically engineered goats is effective in reducing the prevention of perioperative and peripartum thromboembolic events in hereditary AT-deficient patients at optimal dosage. The results of the multicentric trial revealed that there were no AEs observed, when rhAT was administered to patients with hereditary AT deficiency in high-risk situations, including elective surgery,[35] childbirth in cesarean section[34], and to restore heparin responsiveness in heparin-resistant patients scheduled to undergo cardiac surgery necessitating CPB.[32,33] This suggests that rhAT is safe to be administered in patients with hereditary AT deficiency and this product can be substituted for human pdAT for various indications. However, dosage optimization studies are needed. Although the immunogenicity on repeated exposure remains to be evaluated, the safety profile appears acceptable. Because rhAT has a much stronger affinity to heparin, it may enhance the pharmacologic effects of heparin and related drugs. Thus, while rhAT offers a substitute of pdAT, it provides an additional antithrombotic agent that can be combined with heparins to achieve desirable anticoagulant and antithrombotic approaches in various indications.

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