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(REVIEW ARTICLE)

The potential use of recombinant immunotoxin and BCL-2 inhibitors for leukemic stem cell elimination

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Abstract

Leukemia is a type of hematological malignancy caused by the dysfunctional proliferation of leukocyte cells. According to the Global Burden of Disease 2019 report, there were over 643,579 new cases of leukemia cancer worldwide in 2020, killing 334,592 people. Chemotherapy, an important part of the treatment of leukemia, not only harm cancer cells but also harm healthy cells, which can lead to negative side effects. This article examines the possibility for cutting-edge therapy using targeted recombinant immunotoxins and BCL-2 inhibitors as targeted therapy against leukemic stem cells. Recombinant immunotoxin is a protein-based therapeutic agent composed of toxins combined with specific antibodies or other molecules such as growth factors, cytokines, or toxins. Diphtheria Toxin (DT) is the most commonly used toxin because it is easily expressed, making the toxin easier to take. The toxin kills cancer cells by inhibiting enzymatic cell protein synthesis. While immunotoxins enhance therapeutic outcomes by targeting leukemia stem cells, their efficacy can be compromised in certain patients due to apoptosis resistance mediated by proteins like BCL-2. The presence of anti-apoptotic protein BCL-2 enables cell survival even after exposure to immunotoxins. Consequently, combining recombinant immunotoxins and BCL-2 inhibitors holds promise for eradicating LSC and preventing relapse.

Keywords: Leukemia; Recombinant immunotoxin; Leukemic stem cells; BCL-2 inhibitors; Cancer

1. Introduction

Leukemia is a type of hematological malignancy caused by the dysfunctional proliferation of leukocyte cells. Leukemia is classified as acute leukemia or chronic leukemia based on the rate of proliferation, and it can also be classified as myelocytic or lymphocytic based on the origin of the cells. Until now, leukemia was a disease with a high rate of morbidity and mortality, making it a significant public health concern.[1] According to the Global Burden of Disease 2019 report, there were over 643,579 new cases of leukemia cancer worldwide in 2020, killing 334,592 people.[2] This statistic shows that leukemia accounts for 3.4% of all cancer cases and 3.8% of all cancer deaths. By 2030, this number is expected to increase to 720,168 new leukemia incident cases and 367,804 leukemia deaths.[3]

Leukemia treatment and management are difficult. Chemotherapy is an important part of the treatment of leukemia. Chemotherapy drugs, on the other hand, not only harm cancer cells but also harm healthy cells, which can lead to negative side effects. Chemotherapy side effects have an impact on the physical health and quality of life of people with leukemia.[4] Therefore, this article examines the possibility for cutting-edge therapy using targeted recombinant immunotoxins and BCL-2 inhibitors as targeted therapy against leukemic stem cells in the hopes that they can be employed as a cutting-edge therapy for leukemia.

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2. The Role of Recombinant Immunotoxins in Leukemic Stem Cell Elimination

Leukemic stem cells (LSC) are a type of cell that can cause leukemia to develop.[5] Leukemia develops as a result of an uncontrolled ability of LSC proliferation and differentiation to renew itself. LSC are derived from normal hematopoietic stem cells (HSC) that undergo STAT5 activity changes mediated by IL-3. The STAT5 pathway is involved in the regulation of a hematopoietic GTPase (RhoH).[6] A decrease in RhoH levels causes an increase in the expression of the IL-3α receptor (IL-3Rα), also known as CD123 as a surface marker of LSC. CD123 is far more abundant in LSC than in HSC. In fact, studies have shown that CD123 is not expressed in HSC. Multiparameter flow cytometry was used to investigate the increase in IL-3R expression. Multiparameter flow cytometry is a technique for determining the physical and chemical properties of individual cells. This tool can identify a specific type of cell even when it is mixed with other cell populations. Sorted cells can be separated and studied separately in further research.[7]

LSC was first identified in AML. According to reports, 45% of AML patients had an overexpression of CD123.[8] Conventional AML therapy consists of a combination of surgery, chemotherapy, and radiotherapy, all of which have low specificity and thus kill both cancer and normal cells. As a result, traditional therapy has numerous side effects. Conventional therapy also frequently leaves LSC with a high risk of recurrence. Recent research suggests that CD123 expression can be used as a marker of minimal residual disease (MRD). MRD is defined as the persistence of a small portion of LSC after therapy administration that is not detectable histopathologically. MRD, on the other hand, can be investigated using flow cytometry of bone marrow cells.[9]

Targeted therapy as a cancer treatment will improve outcomes while reducing toxicity and side effects. The use of cell surface marker molecules to identify and separate cancer cell targets from normal cells can be used as a targeted therapy approach in cancer. CD123 is a therapeutic target for killing LSC in AML due to increased IL-3Rα expression. Because clonal evolution of LSC has no effect on CD123 expression as a cell surface marker, it can be used as a therapeutic target at various stages of AML, including initial diagnosis, MRD, and recurrence.[10]

One of the potential target therapy strategies is recombinant immunotoxin (RIT). Recombinant immunotoxin is a protein-based therapeutic agent composed of toxins combined with specific antibodies or other molecules such as antibody fragments, cell penetrating peptides (CPPs), growth factors, cytokines, or ligands.[11] To create first-generation immunotoxins, the entire toxin chain is combined with specific antibodies. However, due to the fact that this immunotoxin can bind to other normal cells, its specificity is low. Understanding the structure of the toxin aids in the improvement of the design of the first generation of immunotoxins by producing a second generation of immunotoxins using chemical bonding and recombinant DNA techniques. Toxins in RIT kill cancer cells, whereas antibodies or other specific molecules bind to specific proteins expressed on the surface of cancer cells.[12]

Toxins derived from bacteria, fungi, or plants can be used in RIT in a variety of ways. However, Diphtheria Toxin (DT) is the most commonly used toxin because it is easily expressed, making the toxin easier to take. Furthermore, DT has a high cancer cell elimination activity with few side effects.[11] *Corynebacterium diphtheriae* produces DT, a single protein chain with 535 amino acids. There are three domains in DT. The A domain (catalytic domain) is made up of 1-193 amino acid chains, the T domain (transmembrane domain) is made up of 194-481 amino acid chains, and the B domain (binding domain) is made up of 482-535 amino acid chains.[13]

The toxin kills cancer cells by inhibiting enzymatic cell protein synthesis. In eukaryotic cells, Domain A inhibits protein synthesis. The T domain aids in the translocation of the A domain from the endosome to the cytosol, while the B domain aids in the binding of DT to its receptor. The portion of the toxin that is not required to kill cancer cells is removed. The remaining toxin fragments are linked to specific molecules, preventing them from binding to normal cells.[14] In RIT, which is used to treat AML, the B DT domain is replaced with IL-3, which binds to CD123. Endocytosis occurs via a receptor-mediated endocytosis mechanism when RIT binds to CD123 via IL-3, bringing DT to the endosome. The pH of the endosomal acid causes a change in the shape of the T domain, exposing the A domain to the cytosol.[14],[15]

The A domain then binds to nicotinamide dinucleotide (NAD) in the cytosol. At the post-translational stage, the A domain transfers adenosine diphosphate ribosyl (ADPR) from NAD to elongation factor 2 (EF-2) into diphthamide residues. This causes irreversible EF-2 inactivation and inhibits protein synthesis, resulting in cell death.[11]

3. The Use of BCL-2 Inhibitors and Immunotoxins in Combination

As previously explained, design features of immunotoxin improve therapeutic outcomes by eliminating LSC. Several studies, however, have found that immunotoxin therapy is less effective in some AML patients. The presence of a

resistance mechanism against apoptosis, including the presence of pro-survival proteins such as the Bcl-2 protein, is one of the reasons for the failure of immunotoxin-induced cell death. The presence of the anti-apoptotic protein Bcl-2 allows cells to survive even in unfavorable conditions, in this case despite being exposed to immunotoxins.[16]

As previously stated, the presence of LSC in the circulatory system of AML patients during and after treatment frequently causes recurrences.[16] Many current studies are focusing on recognizing the molecular mechanisms of LSC and understanding how they differ from normal HSC in order to avoid recurrence of AML cases. The difference in the regulation of reactive oxygen species (ROS) production in LSC and HSC is one of the main differences that could be used as a therapeutic target.[17]

Organic and inorganic molecules with an odd number of electrons on their outer surface are known as ROS. ROS molecules are frequently linked to O_2 free radicals.[18] When a molecule oxidizes in a metabolic process, oxygen is reduced, resulting in an increase in ROS intermediates such as hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), and superoxide anion ($•O_2^-$). Because of the presence of an unpaired outer valence electron, this molecule is highly reactive and can cause a chain reaction between molecules, resulting in oxidative damage.[19]

ROS are produced in many in vivo reactions under normal conditions, including by-products of activated phagocytic cells, mitochondrial respiration, and the nicotinamide adenine dinucleotide phosphae (NADPH) oxidase reaction.[20] One of the primary sources of ROS in most mammalian cells is energy production via mitochondria. When adenine triphosphate (ATP) is produced via the mitochondrial oxidative phosphorylation mechanism, the substrate is oxidized by various enzymes found in the mitochondrial membrane.[21]

The process of forming ATP via the electron transport chain is known as oxidative phosphorylation. In the mechanism of the electron transport chain, there are electron carriers named complexes. These electron carrier complexes are embedded in the inner mitochondrial membrane, where electrons can move in and out to generate energy. The cytochrome-c oxidase molecule, also known as complex IV, is involved in the final step of this process. This complex transfers electrons to oxygen (O_2), resulting in the molecule splitting and the formation of two oxygen atoms (O).[22]

The electrons transferred to the oxygen atom make it electronegative and capable of attracting two proton atoms, such as H^+ ions, to form two H_2O molecules. According to this description, it takes four electrons to convert O_2 to H_2O. When the number of electrons transferred to oxygen was less than four, ROS production increased. If oxygen accepts only one electron, the oxygen atom becomes $•O_2^-$. Meanwhile, if the oxygen atom accepts two electrons, it will form H_2O_2. Meanwhile, the oxygen atom will form •OH if it accepts three electrons.[23]

Embryonic stem cells have small immature mitochondria. When cells differentiate, however, mitochondria elongate and undergo a maturation process in order to meet the production of ATP.[24] Similarly, HSCs (stem cells) have mitochondrial activity and a low membrane potential. This is one of the most important properties for protecting stem cells from increased ROS. As a result, HSC stem cells generate energy via anaerobic glycolysis rather than mitochondrial oxidative phosphorylation.[25] Although anaerobic glycolysis is less efficient than mitochondrial oxidative phosphorylation, it is very effective at limiting HSC ROS production.[26]

Low ROS levels in HSCs are required to regulate stem cell renewal capacity and maintain cell genome stability. More differentiated cells, such as HPCs and precursor cells of various hemopoietic progeny, produce more ROS than HSCs. The high level of ROS in differentiated cells reflects oxidative metabolism activity in these cells. Although an increase in ROS levels beyond normal limits can cause cell damage, an increase in ROS levels within normal limits can induce HSC proliferation, differentiation, and maturation.[27]

Initially, it was thought that HSCs had fewer mitochondria than other cells, resulting in less excess ROS production. Nonetheless, research has revealed that HSC contain a large number of mitochondria. However, the majority of these mitochondria are dormant. This finding supports the theory that oxidative metabolism is not the primary source of energy in HSC.[28]

ROS levels that exceed the threshold, as previously stated, are toxic to HSC. Elevated ROS levels have also been linked to leukemia development. High levels of ROS frequently cause numerous mutations in HSC DNA, including DNA methylation and chromatin modification, resulting in the formation of pre-leukemic HSC. If these cells are continuously exposed to ROS and undergo mutations, they will eventually transform into LSC.[29]

A study in rats with defects in the ataxia-telangiectasia mutated (ATM) pathway that, after being observed for some time, had a failure of hematopoiesis function is one of the main pieces of evidence demonstrating the toxicity of oxidative

stress to HSC. When the ATM pathway fails, ROS production in HSC increases.[30] ROS can cause mitochondrial DNA damage as well as damage to mitochondrial lipids and proteins, which can cause HSC senescence and apoptosis. ROS can also oxidize and damage the outer and inner mitochondrial membranes, resulting in respiratory chain dysfunction and cell death.[31]

Changes in the structure and permeability of the mitochondrial outer membrane are thought to be the cause of mitochondrial dysfunction caused by ROS accumulation. Furthermore, the presence of the mitochondrial permeability transition pore complex (MPTP) influences mitochondrial-mediated cell apoptosis.[32] The MPTP complex is made up of cyclophilin D, a mitochondrial matrix molecule that is linked to an adenine nucleotide translocator on the mitochondrial inner membrane. Increased levels of free radicals activate MPTP and increase the permeability of the inner membrane, allowing molecules less than 1.5 kDa in size, including protons, to enter the mitochondrial matrix and disrupt mitochondrial function.[33]

If this process is allowed to continue, the mitochondria will swell due to osmosis. The combination of MPTP activation and increased mitochondrial inner and outer membrane permeability results in the release of pro-apoptotic proteins into the cytosol, including cytochrome c, apoptosis inducing factor (AIF), and endonuclease *G* (endoG). The release of pro-apoptotic proteins eventually results in cell apoptosis.[34]

Considering this, controlling ROS levels in stem cells is one of the most important aspects of cell survival. HSCs have several mechanisms to counteract increased ROS and prevent oxidative stress, including the use of different metabolic pathways to reduce ROS production and the presence of an anti-ROS protein that encodes the ATM gene. The presence of low molecular weight reducing peptides like glutathione, thioredoxin, and NADPH, as well as antioxidant enzymes like catalse, superoxide dismutase enzymes, and glutathione peroxidase, contribute to this mechanism.[35] HSC stem cells also develop an autophagy mechanism called mitophagy, which can reduce the number of ROS-producing mitochondria.[36]

Another mechanism of HSC protection against ROS is the transcription factor Forkhead O (FoxO), which can activate antioxidant enzymes required for ROS detoxification as well as enzymes capable of repairing ROS-induced DNA damage. Catalase and superoxide dismutase are two of these enzymes. Animal studies have shown that removing the transcription factors FoxO1, FoxO3, and FoxO4 from hematopoietic cells increases ROS levels in HSCs, which can be corrected by administering N-acetyl-L-cysteine.[37]

Meanwhile, LSC is a stem cell with nearly the same properties as HSC, namely the ability to divide continuously in order to continue producing malignant cells. This has been demonstrated in studies on experimental animals infected with leukemia and given LSC through the animal's vein.[38] In contrast to the processes and mechanisms used by HSC to generate energy, LSC uses the integrity and metabolism of mitochondria, as well as oxidative phosphorylation mechanisms, to generate energy for survival.[39]

Inhibiting complex IV in mitochondria results in LSC cell death, proving the use of mitochondria as the primary energy producer for LSC.[40] Other studies have found that when LSC mitochondrial activity is inhibited, the cells cannot survive because they are unable to perform glycolysis to produce energy. Furthermore, LSC death was observed when an inhibitor molecule inhibiting complex I of the mitochondrial electron transport chain was added.[41]

ROS levels in LSC have increased due to their reliance on mitochondrial oxidative phosphorylation. Furthermore, activation of the FLT3-ITD pathway has been shown to increase ROS production in most LSCs with DNA damage and instability. A rise in ROS in LSC is also linked to a drop in p22 and an increase in STAT5.[42]

As previously stated, many enzymatic systems, such as xanthine oxidoreductase (XOR), cyclooxygenase (COX), lipooxygenase, and NADPH oxidase (NOX), contribute to increased ROS levels in cells.[19] The NADPH oxidase enzyme class is one of the enzyme systems that produces the most ROS of these enzymes. The NADPH oxidase group consists of six enzymes: NOX1, NOX2, NOX3, NOX4, and NOX5. According to research, there is an increase in the activity of the NADPH oxidase enzyme in LSC, which contributes to the increase in LSC ROS.[43]

Because mitochondrial DNA is not packaged in chromatin, it is more vulnerable to oxidative damage than nuclear DNA. Furthermore, the lack of introns in mitochondrial DNA increases the likelihood of mutations occurring in coding regions of the DNA. As a result, increased ROS production in LSCs may threat mitochondrial DNA stability. Oxidative stress can result in mutations and DNA damage, which can lead to an increase in ROS production.[44]

To survive in the presence of high levels of ROS, LSC are likely to rely on factors that inhibit apoptosis and programmed cell death, or factors that protect cells from damage caused by oxidative stress. Several recent studies have found that LSC are increasingly reliant on the BCL-2 anti-apoptotic mechanism. LSC reliance on the BCL-2 anti-apoptotic mechanism can be used to eliminate LSC and prevent leukemia recurrence.[45]

BCL-2, an anti-apoptotic protein, is an important regulator of mitochondrial physiology and cellular stress response. BCL-2 is an anti-apoptotic protein that prevents increased mitochondrial outer membrane permeability by interacting with the pro-apoptotic proteins BAX and BAD.[46] BCL-2 has another function that influences mitochondrial activity by regulating pro-oxidative and anti-oxidative processes. BCL-2 can interact with the mitochondrial complex IV COX-Va subunit, which is part of the electron transport chain. Complex IV interaction can improve mitochondrial function. BCL-2 can also increase the transport of the anti-oxidant glutathione to mitochondria.[47] Accordingly, high levels of BCL-2 can reduce mitochondrial membrane permeability by reducing pyridine nucleotides.[48]

In short, BCL-2 can maintain mitochondrial stability and prevent LSC apoptosis in the presence of high levels of ROS by preventing a decrease in mitochondrial membrane potential and an increase in membrane permeability. This can be accomplished by increasing mitochondrial activity and acting as an antioxidant to compensate for increased ROS. As a result, BCL-2 inhibition has the potential to be used as a strategy for LSC eradication. According to research, BCL-2 inhibition affects LSC survival and can improve the efficacy of chemotherapy drugs.[49]

Other research has found that LSC are more sensitive to BCL-2 inhibition than other cells, including HSC, due to the high expression of BCL-2 in LSC. Furthermore, a study comparing the effects of BCL-2 inhibitors on LSC cells with low ROS levels versus LSC cells with high ROS levels in AML discovered that BCL-2 inhibitors were more sensitive to LSC cells with low ROS levels.[41] As a result, the mechanism of anti-apoptotic inhibition of BCL-2 has the potential to be used in the eradication and prevention of LSC.

4. Conclusion

The high morbidity and mortality rates of leukemia throughout the world make it critical to treat this disease. Chemotherapy is the primary treatment for leukemia, but it also damages healthy cells, which can result in a variety of side effects. This encourages the development of more effective leukemia treatments, such as targeted immunotoxin recombinants and BCL-2 inhibitors that target leukemic stem cells (LSC). Recombinant immunotoxin is a protein-based therapeutic agent composed of toxins combined with specific antibodies or other molecules such as antibody fragments, cell penetrating peptides (CPPs), growth factors, cytokines, or ligands. Toxins derived from bacteria, fungi, or plants can be used in RIT in a variety of ways. However, Diphtheria Toxin (DT) is the most commonly used toxin because it is easily expressed, making the toxin easier to take. The toxin kills cancer cells by inhibiting enzymatic cell protein synthesis.

Although the design features of immunotoxins improve therapeutic outcomes by eliminating LSC, several studies have shown that immunotoxin therapy is less effective in some AML patients. This is due to the mechanism of apoptosis resistance, which includes the presence of *pro-survival* proteins such as the BCL-2 protein. The presence of the anti-apoptotic protein BCL-2 allows cells to survive even in unfavorable conditions, in this case despite exposure to immunotoxins. As a result, the mechanism of anti-apoptotic inhibition of BCL-2 with recombinant immunotoxins has the potential to be used in eradicating LSC and preventing recurrence. Further research into the efficacy of using recombinant targeted immunotoxins and BCL-2 inhibitors as leukemia therapy can begin immediately, allowing clinical trials to begin, which can then be used by the public in the future to reduce leukemia mortality.

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