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Polymer-conjugated inhibitors of tumor necrosis factor-α for local control of inflammation

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Burns, chronic wounds, osteoarthritis, and uveitis are examples of conditions characterized by local, intense inflammatory responses that can impede healing or even further tissue degradation. The most powerful anti-inflammatory drugs available are often administered systemically, but these carry significant side effects and are not compatible for patients that have underlying complications associated with their condition. Conjugation of monoclonal antibodies that neutralize pro-inflammatory cytokines to high molecular weight hydrophilic polymers has been shown to be an effective strategy for local control of inflammation. Lead formulations are based on antibody inhibitors of tumor necrosis factor-α conjugated to hyaluronic acid having molecular weight greater than 1 MDa. This review will discuss fundamental aspects of medical conditions that could be treated with these conjugates and design principles for preparing these cytokine-neutralizing polymer conjugates. Results demonstrating that infliximab, an approved inhibitor of tumor necrosis factor-α, can be incorporated into the conjugates using a broad range of water-soluble polymers are also presented, along with a prospectus for clinical translation.

Types of Conditions Requiring Local Control Over Inflammation

Systemic autoimmune diseases, such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease, have been a central focus of the pharmaceutical industry.[1,2] While there are no cures yet, and millions of patients still face significant health challenges and decreases in quality of life, extensive research and drug development have resulted in the development of powerful therapies capable of ameliorating symptoms. In contrast, there are a number of conditions characterized by local, intense inflammation that lack effective treatments. Some are associated with acute injuries, such as burns, others are due to underlying chronic disease states, such as osteoarthritis, and others appear to result from chronic inflammation due to underlying disease states, as in diabetic ulcers, or complications from treatment for other conditions, as in oral mucositis stemming from radiation treatment.

Inflammatory conditions can be categorized along two axes, as shown in **Figure 1**. The first is whether the condition results from an acute injury to an otherwise healthy individual or is due to an underlying disease state that can be chronic in nature. The second is whether the main inflammatory processes are local or systemic in nature. Burn injuries over relatively small areas are an example of an acute, local inflammatory state while rheumatoid arthritis is an example of a chronic, systemic inflammatory condition. Diabetic ulcers and Crohn's disease are intermediate in this classification scheme since they have roots in underlying chronic, systemic diseases but the symptoms that necessitate treatment tend to be local in nature. While this scheme is a gross oversimplification, it does provide a framework for analyzing conditions that may be amenable to therapies that are designed to act locally. In principle, both acute and chronic conditions could be treated locally, but more effective therapeutic strategies must be developed.

Anti-Inflammatory Drugs

The most potent anti-inflammatory drugs have been primarily developed to treat autoimmune diseases, such as rheumatoid arthritis or psoriasis. In the case of psoriasis, there is a hierarchy of treatment options depending on the severity of the condition.[3,4] For mild psoriasis, topically applied steroid creams can be effective at managing the plaques that form. The effects of hydrocortisone, a steroid used widely in relieving symptoms of inflammation, include downregulation of pro-inflammatory cytokine production, especially interleukin-2, and decreased expression of COX-1 and COX-2 enzymes. However, hydrocortisone is absorbed rapidly through skin and epithelial tissues and ultimately results in systemic dosing, with side effects that can include immunosuppression, inhibition of bone formation, impaired wound healing, and a host of other complications. Given its moderate anti-inflammatory activities, the trade-offs associated with its side effects make it unsuitable for extended use. Other classes of small-molecule immune suppressants have been developed, including calcineurin inhibitors,[5] but these also suffer from significant side effects.

In more serious cases of plaque psoriasis, inhibitors of tumor factor-α (TNF-α) are required to push the disease into remission.

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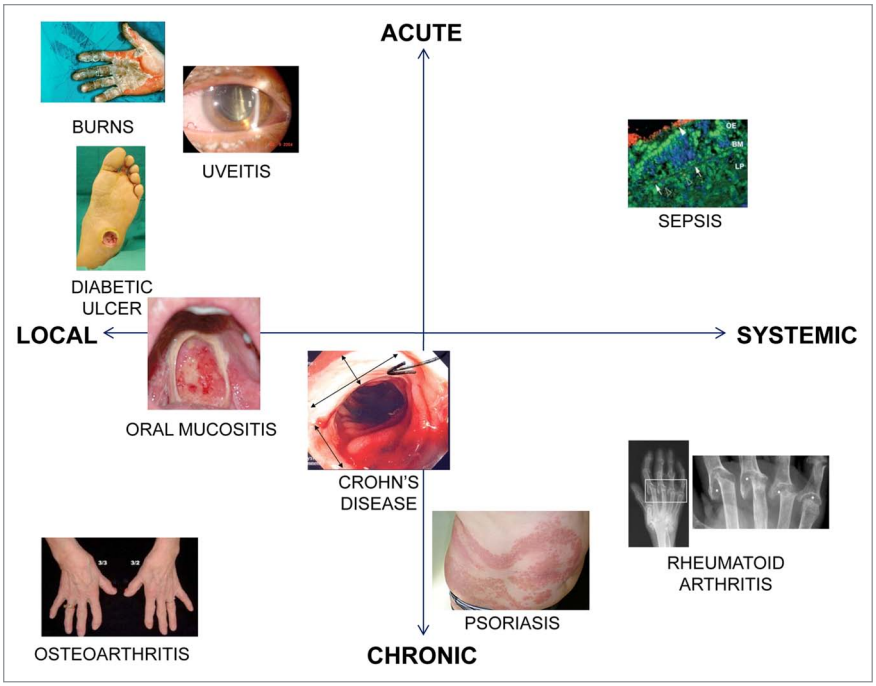

Figure 1. Simplified categorization of inflammatory conditions according to whether the condition is acute or chronic, and whether the inflammatory component has a predominantly local manifestation or is systemic in nature.

TNF-α is a cytokine that is often referred to as the master regulator of the inflammatory response.[6] Numerous clinically approved inhibitors of TNF-α are available, most of which are based on monoclonal antibodies.[7] For example, infliximab is a fusion protein based on a variable domain derived from a mouse antibody against human TNF-α incorporated into a human IgG1, while etanercept is a fusion protein composed of a human receptor fragment for TNF-α fused into an IgG1 construct. Various antibody fragments and peptide inhibitors of TNF-α are also either available clinically or in clinical trials, including certolizumab pegol, a PEGylated antibody fragment. Standard, systemically administered doses for these drugs are approximately 100 mg for adult patients.

Next-generation anti-inflammatory drugs are being developed for more targeted application. These leverage our growing understanding of the pathophysiology of inflammatory conditions, neutralizing specific mediators involved in the development of the disease state. While many signaling molecules are upregulated substantially, not all are therapeutic targets. For example, despite the well established increases in IL-6 levels following thermal injuries, sepsis, and other inflammatory conditions, neutralizing it appears to have mixed therapeutic benefit in pre-clinical models[8] and clinical trials are still ongoing. IL-6 has complex functions in the inflammatory microenvironment, with one general role to serve as a bridge between inflammatory and healing responses, and the window in which neutralizing it is therapeutically effective may be narrow.[9] Examples of new therapeutic agents are neutralizing antibodies against interleukin-12 (IL-12) for treatment of Crohn's disease and interleukin-12/23 for treatment of psoriasis. IL-12 is a cytokine composed of p40 and p19 subunits produced by dendritic cells, macrophages, and other cells that respond to antigen stimulation.[10] It stimulates T cells and induces production of interferon-γ and TNF-α, so while it is not positioned at the apex of inflammatory responses as TNF-α is, it still can play a critical role in signaling pathways of disease pathologies. Early clinical trials of anti-IL-12 in Crohn's disease appeared to demonstrate positive effects compared with placebo treatment.[11] Similarly, IL-23 is a member of the IL-12 family and also contains the p40 subunit. Neutralizing antibodies against IL-12/23 have been shown to be effective in treating psoriasis in early clinical trials.[12] Numerous innovative therapeutics are being developed that leverage our improved understanding of the immunological basis of disease and injury.

Molecular Fluxes and Transport in Inflammatory Responses

In considering molecular transport in inflamed tissues, it is important to understand the fluxes of important signaling molecules involved in mounting inflammatory responses. There are relatively few reported absolute measurements of cytokine concentrations in inflamed tissues, and interactions between cytokines and the extracellular matrix are poorly understood. Burn injuries can be used as a representative example. Pro-inflammatory cytokines in burned tissue can directly stimulate cellular necrosis as well as increase vascular permeability, vasodilation, and production of matrix-degrading enzymes, all of which contribute to continued tissue necrosis in a hypoxic environment. The concentrations of pro-inflammatory cytokines in thermally injured skin have been studied in a mouse model. Schwacha et al. reported a nearly 100-fold increase in TNF-α concentration in burned skin and a nearly 500-fold increase in IL-6 when compared with undamaged skin.[13] Three days following the original injury, TNF-α levels in digested tissue were measured to be 1,500 ng/(μg soluble protein), which is estimated to be 45 μg/mL. Interestingly, TNF-α levels in the wound exudate were only 100 pg/mL, suggesting it is strongly retained in the tissue, either entrained in the extracellular matrix or in a membrane-bound form.

In osteoarthritis, a number of pro-inflammatory cytokines are found in increased concentrations in the synovial fluid compared with healthy joints. These include TNF-α (up to 80 pg/mL compared with < 4 pg/mL), IL-2 (up to 12.5 pg/mL compared with ~0 pg/mL), and IL-1β (up to 27.8 pg/mL compared with ~5 pg/mL). These cytokines stimulate production of matrix-degrading enzymes that result in the irreversible deterioration of cartilage in the joints. As in the case of burns, the concentration of cytokines in the actual tissue is likely much higher than what is recovered in the surrounding fluid, suggesting strong

interactions with the extracellular matrix and potentially hindered molecular transport.

Molecular transport in the extracellular matrix is a critical factor in biochemical signaling pathways and can be used in understanding the evolution of inflammatory states and in designing treatments.[14] Diffusion is an important clearance mechanism, and the time to diffuse a given distance will vary inversely with the diffusion constant. Most cytokines have molecular weights below 100 kDa and diffusion constants that are thought to be rapid enough to conduct extracellular signaling. For example, the diffusion constant of TNF-α in tissue has been estimated to be 2×10^{-7} cm^2/s.[15] Small molecule drugs, such as hydrocortisone, have diffusion constants in buffer solution of order 10^{-6} cm^2/s, but this may be significantly lower in tissue depending on the state of hydration and density of extracellular matrix. Studies of antibody diffusion have shown that the diffusion constant of 6.3×10^{-7} cm^2/s measured in buffer is reduced to 10^{-8}–10^{-9} cm^2/s in healthy and neoplastic tissues.[16] In vascularized tissue, assuming a mean distance to a capillary of 50 μm, a diffusion constant of 10^{-7} cm^2/s conforms to a mean diffusion time of order one hour, and a diffusion constant of 10^{-8} cm^2/s would result in a diffusion time on the order of a day. Once at a capillary, especially one with increased permeability due to the presence of inflammatory mediators, signaling molecules and therapeutic agents can exit the site. While enzymatic degradation and convection-driven clearance processes clearly can be important factors in determining the mean residence time of molecular determinants of inflammatory responses, consideration of diffusive transport can provide estimates used in understanding and designing novel therapeutic approaches. If inflamed tissues produce a cytokine at volumetric rates as high as 10 μg/mL·day, therapeutic agents that are cleared within hours will require frequent application in order for the local concentration to remain in the therapeutic window.

Approaches to Local Control of Inflammation

There are three main approaches to local delivery of anti-inflammatory therapeutic agents: (1) frequent administration of low concentrations; (2) controlled release from concentrated drug depots; (3) sustained delivery of low concentrations. An example of the first approach is topical application of corticosteroid creams for atopic dermatitis. These creams usually contain 1% active ingredient, such as hydrocortisone or betamethasone, and are administered 1–4 times per day. This approach is most commonly used for small molecule drugs and finds wide application commercially. The permeability and diffusion constants of hydrocortisone and other small molecule drugs in skin varies widely according to moisture content and drug concentration, but permeability values of 1.6×10^{-5} cm/h have been reported for hydrocortisone through the stratum corneum while progesterone can be as low as 1.3×10^{-2} cm/h.[17] The clearance rate of dilute hydrocortisone solutions from human dermis has been estimated to be 0.1 mL/h, suggesting the mean residence time of these drugs in the skin is dominated by transport through the stratum corneum and clearance from the dermis is rapid.[18]

Infliximab has been tested in a pilot clinical trial investigating whether topical application improves healing of chronic wounds.[19] The preliminary results were encouraging, although patients received solutions containing 10 mg/mL infliximab, which could result in a dose similar to that via injection.

Delivery of anti-inflammatory drugs from depots, such as degradable polymer microspheres, has been investigated extensively.[20] This approach has been demonstrated in pre-clinical studies for intra-articular, colonic, and topical delivery using particles based on polylactide, chitosan, lipids, and other polymer constructs that erode in biological environments.[20-22] While drugs may diffuse rapidly from the site, the polymer constructs are designed to provide steady doses over hours or even days designed to provide more effective treatment than periodic administration. Solid polymer microspheres often provide a burst of drug delivery from the outer surface of the particles followed by extended steady release but the overall release profile is extended to hours or days compared with minutes in the case of direction therapeutic delivery.[23,24] Clinical translation of this approach has not yet been reported in the case of inflammatory conditions, but the area appears to be ripe for further development as our understanding of structure-property relationships leads to increasingly sophisticated methods for controlled release of therapeutic agents.[25]

The third approach is hindering transport of the therapeutic agent from the site of application. Our lab has developed a strategy for doing this with therapeutic antibodies against pro-inflammatory cytokines, and the remainder of this review will describe the strategy and its potential applications.

Sustained Antibody Delivery through Polymer Conjugation

Antibody conjugation to high molecular weight hydrophilic polymers has been developed as a means for providing sustained delivery of inhibitors of TNF-α and other mediators of inflammation to sites of injury or disease. The basis for the approach is slowing diffusion of the antibody by tethering it to a high molecular weight biocompatible polymer that slows diffusion. Published examples include conjugation of rodent IgG1 neutralizing antibodies against TNF-α and IL-1β to hyaluronic acid (HA; M_w = 1.6 MDa) and carboxymethyl cellulose (CMC; M_w = 100 kDa).[26] Carbodiimide chemistry was used in activating carboxylic acid groups on the HA and CMC to react with pendant amine groups on the antibody and form amide linkages between antibody and polymer, shown schematically in **Figure 2A** with HA as an example. This is a non-site-specific coupling reaction, but the equilibrium binding constant, measured using an optical interferometry biosensor, is similar to the non-conjugated antibody. Shown in **Figure 2B** are association and dissociation curves for IgG1 against human TNF-α, and the same antibody conjugated to HA, both having K_D values of 120 pM. In contrast, non-site-specific conjugation of aldehyde-terminated PEG having molecular weight 80 kDa at a 1:1 molar ratio abolished TNF-α binding, only resulting in a slowly decreasing baseline that may be due to dissociation of weakly

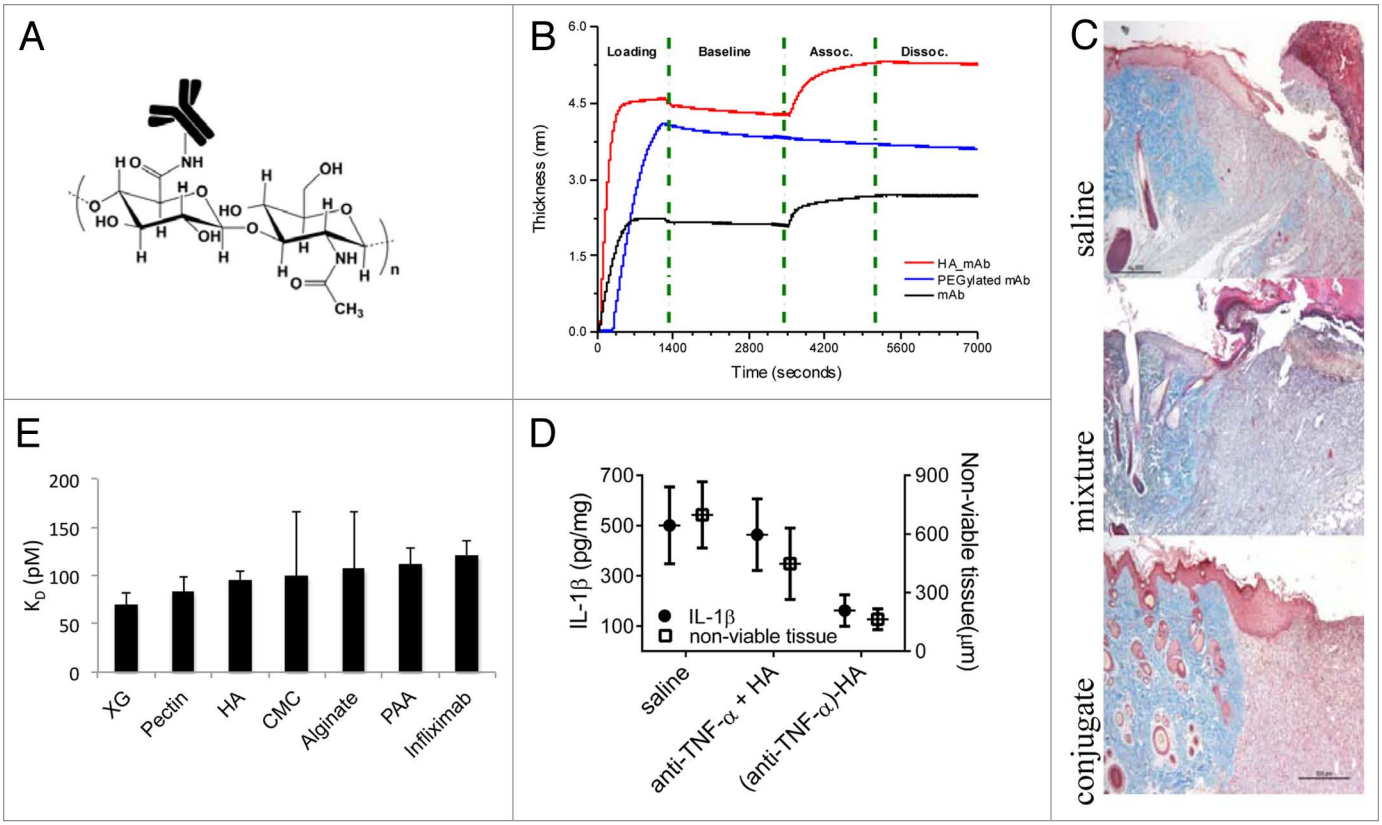

Figure 2. (**A**) Schematic representation of mAb-HA conjugate formed through an amide linkage. (**B**) Measurement of binding (Assoc.) and release (Dissoc.) of TNF-α by mAb, PEGylated mAb, and mAb-HA conjugate using ForteBio Octet. (**C**) Trichrome-stained tissue sections from partial-thickness burn experiments in rat model showing day 7 results following treatment with saline, anti-TNF-α and HA mixture, or (anti-TNF-α)-HA conjugate. (**D**) Correlation between concentration of IL-1β in burn tissue and secondary necrosis measured 7 d following original injury. (**E**) Equilibrium binding constant KD measured for infliximab and conjugates formed with xanthan gum (XG), pectin, HA, carboxylmethylcellulose (CMC), alginate, and polyacrylic acid (PAA).

bound PEGylated antibody. PEGylation is known to affect binding through strong protein-polymer interactions, making design of PEGylated therapeutics a complex process. Certolizumab pegol is PEGylated at a pendant thiol group in the hinge region of the protein using a branched PEG having two 20 kDa tails. It is remarkable that non-site-specific conjugation of 1.6 MDa HA to monoclonal antibodies preserves binding affinity for TNF-α when functionalization using an 80 kDa PEG reduces binding to unmeasurable levels. In both cases the sites of conjugation are reactive amines, such as lysine residues, but we propose that strong antibody-PEG interactions shroud the binding domains while the HA allows interactions with the antigen.

The HA conjugates against IL-1β and TNF-α have been tested in a rat incisional wound model and were shown to modulate early inflammatory responses at total antibody doses of 100 μg.[27] Quantification of macrophage infiltration and phenotype expression 4 d following injury demonstrated a significant reduction in the number of cells expressing the CD68 pan-macrophage marker and CCR7, the marker for classically activated macrophages. This indicates that antibody doses that are 1,000-fold lower than injected systemically in adult humans can local modulate responses to injury. However, delivery of the polymer conjugates in a crosslinked form as a hydrogel essentially abolished anti-inflammatory activity, indicating that the conjugates need to be incorporated into the extracellular matrix in order to function.[28] In subsequent, unpublished studies, conjugates containing only antibodies against IL-1β at identical doses were found to be ineffective at altering responses to injury, but conjugates that target only TNF-α achieved similar results as the mixture of antibodies against these two cytokines. While both cytokines are among the first to be upregulated following injury,[29] TNF-α inhibition is used broadly while IL-1β inhibitors find limited use clinically.[30] This points to an important strategy in regulating inflammatory processes in acute injuries: targeting downstream cytokines or those that play supporting roles in establishing the inflammatory microenvironment is less likely to be effective than targeting those centrally involved.

The therapeutic potential of anti-TNF-α conjugated to HA was tested in a rat partial-thickness burn model. Rodent burn models recapitulate many of the important early inflammatory responses to these intense injuries that occur in humans,[31] providing a useful model for pilot studies on novel therapeutic approaches with immunological mechanisms. In rodents, as in humans, intense inflammatory responses can promote secondary necrosis in a process known as burn progression.[32] Representative tissue sections following trichrome staining are

shown in **Figure 2C**. Rats in this study that received saline treatment lost an additional 700 μm of dermal tissue while those that received three doses of (anti-TNF-α)-HA conjugate containing 50 μg antibody each had a 70% reduction in secondary necrosis. Interestingly, conjugates against IL-6 may actually increase secondary necrosis compared with delivery of the conjugate,[33] as did non-conjugated mixtures of anti-TNF-α and HA, which was tested in a follow-up study.[34] Conjugation of anti-TNF-α to HA appears to provide potent anti-inflammatory effects at the site of injury that resulted in reduced secondary necrosis, as evidenced by the correlation between IL-1β concentrations in burned tissue with measured extent of secondary necrosis (**Fig. 2D**).

Based on comparing PEG to HA, it appears that negative charge is an important factor in the conjugation polymer for retaining antibody binding when non-site-specific conjugation is used. To investigate the possibility of incorporating clinical inhibitors of TNF-α, we explored conjugation of infliximab to a diversity of negatively charged polymers and biopolymers. The results are summarized in **Figure 2E**. The non-conjugated inhibitor was measured to have a K_D value of 121 pM, higher than its reported value of 28 pM[35] but still consistent with tight antigen binding. Conjugation of infliximab with xanthan gum (XG), poly(acrylic acid) (PAA), pectin, and HA all resulted in conjugates with similar or improved binding to the bare antibody. Carboxymethylcellulose (CMC) and sodium alginate (ALG) gave inconsistent results, suggesting these polymers may interfere with binding, since it was the association rate constant k_{on} that had the largest variability. Of the polymers tested, only HA has significant intrinsic biological activities, promoting cell motility and acting as a damage-associated molecular pattern upon degradation.[36] Further research will be necessary to understand the influence of polymer chemistry and biochemistry on conjugate activities, but the approach utilizing negatively charged polymers in conjunction with antibody-based inhibitors appears to be quite general.

Prospectus

Challenges in developing antibody-polymer conjugates include validating them for use in treatment of autoimmune diseases that present with local manifestations of persistent inflammatory states, such as psoriasis and Crohn's disease. In addition, injuries in healing-impaired models, such as rodent models of diabetes, will provide an important test of whether intrinsic healing responses are sufficient to repair injuries if intense inflammation is modulated. Finally, a better understanding of the interplay between intrinsic biochemical activities of polymers and biopolymers used to conjugate to antibodies and their degradation rates will provide insight into the pharmacokinetics of this drug-delivery strategy and its overall biological activities. Ultimately, this therapeutic approach will need to be tested in humans to determine whether local cytokine neutralization is an effective treatment strategy. Inhibitors of TNF-α are currently the most promising formulation, but rapid development of next-generation inhibitors of mediators of inflammation could allow even more targeted treatment. Combined with improvements in polymer delivery vehicles, potentially offering time-dependent or stimulus-responsive delivery, and this approach could treat conditions for which there are very few effective options currently.

Disclosure of Potential Conflicts of Interest

NRW has started a company to commercialize aspects of this research and acknowledges a potential conflict of interest.

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