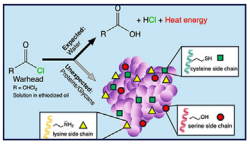

Recent Developments and Future Directions in Image-Guided Chemistry

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ABSTRACT

Transcatheter embolotherapies have undergone tremendous evolution and development since the initial reports 5 decades ago. Imaging has likewise advanced with the advent and refinement of computed tomography (CT) and magnetic resonance (MR) imaging. Despite these technical improvements, viable tumor is found in the majority of embolotherapy treated tumors examined under the microscope. Thermoembolization is a novel approach under development that uses in vivo chemistry to address this problem. Relying on basic chemical principles of reactivity, exciting possibilities are emerging for more effective treatments. Furthermore, the serendipitous discovery of unexpected chemical reactions has pointed to a new area of investigation. The authors introduce a unique paradigm, expanding targeted, image-guided in vivo chemistry beyond initial destructive interventions to nondestructive ones, particularly protein modifications. An entirely new world of opportunities is opening for collaboration between interventional radiologists, chemists, and molecular biologists.

ABBREVIATIONS

CT = computed tomography, MR = magnetic resonance

Transcatheter therapies for unresectable liver cancer are familiar territory to interventional radiologists. These methods have evolved substantially since the initial reports of embolization, intra-arterial drug infusion, and combinations of the 2 strategies (1,2). Ethiodized oil emulsions were typically followed by embolization using flocculent polyvinyl alcohol particles of irregular size and shape, although a host of other materials have been employed as well. The past 2 decades have born witness to technical advances on many fronts for embolic materials. Among these are the development of microspheres with narrowly calibrated size ranges, drug-eluting beads, imageable beads, radioembolization, and, most recently, resorbable materials and conformable materials (3–9). At the same time, technology for computed tomography (CT) and magnetic resonance (MR) imaging has advanced, and reporting standards and imaging criteria such as the modified Response Evaluation Criteria in Solid Tumours and European Association for the Study of the Liver continue to be refined (10). Despite these advances and reports of complete response by imaging, viable tumor persists in the majority of treated tumors examined under the microscope (11–16).

This backdrop suggested that a fundamentally different approach could be valuable. The problem of incomplete treatment provided the motivation to explore new territory merging embolotherapy with reactive chemistry. Taking note that water makes up an estimated 60%–70% of the body, a potential strategy emerged: exploit water in the tissue as a reagent. The authors hypothesized that due to its abundance, water would react with a suitable molecule. In the first iteration of this idea, thermoembolization, the goal is tissue destruction. Myriad compounds could serve in this role, provided that they incorporate a suitably reactive, water-sensitive functional group at 1 end to serve as what chemists refer to as the warhead.

The authors' initial choice of functional group for the warhead was an acid chloride. The acid chloride functional group is likely much less familiar to clinicians than the far more stable amides and esters, which are ubiquitous in peptide bonds and drugs. An acid chloride has a chlorine atom bonded to a carbonyl instead of an amine or alkoxy group as found in amides and esters. This change makes it much more reactive, and this reactivity can be extremely useful. Acid chlorides are known to release heat energy upon encountering water, and in addition, reaction with water would result in production of acid as shown in **Figure 1**. Protein conformation and, thus, function can be altered by heat and pH, so both of these effects would help achieve the desired result of necrosis. Early results in this area have been highly encouraging (17). The authors have successfully shown proof of concept in vivo (18) and that systemic exposure is very low (19,20). **Figure 1** illustrates the upper, expected pathway and reaction products in an

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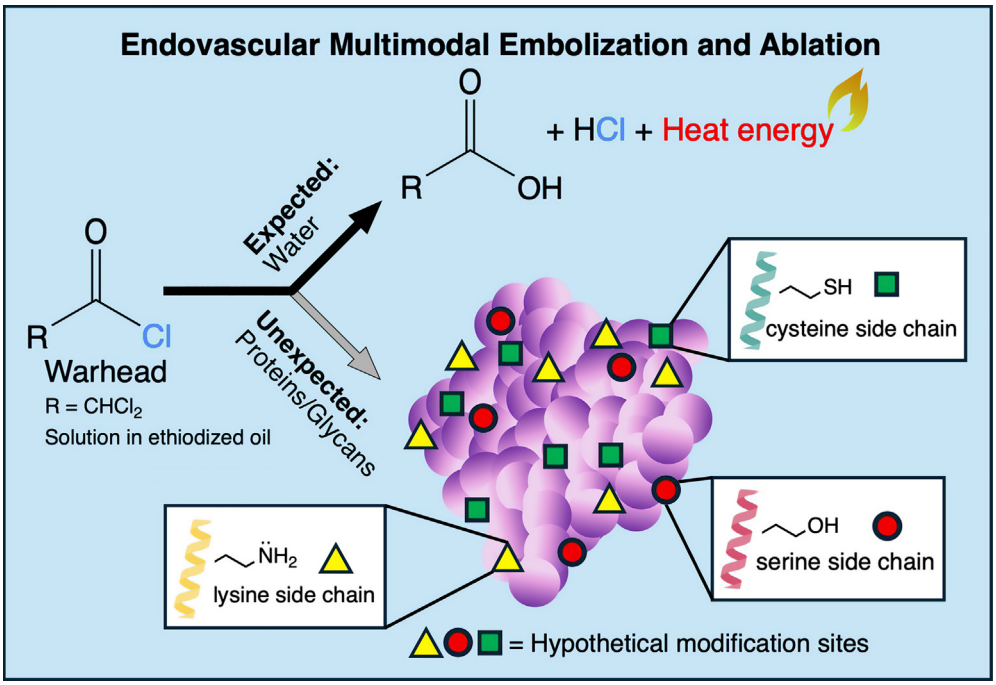

Figure 1. Thermoembolization reaction and effects in pig liver. Expected reaction of acid chloride upon hydrolysis yields acid and heat to cause denaturation and necrosis. Unexpected reaction illustrates covalent modification of biological macromolecules at amino acid side chains such as lysine, cysteine, and serine.

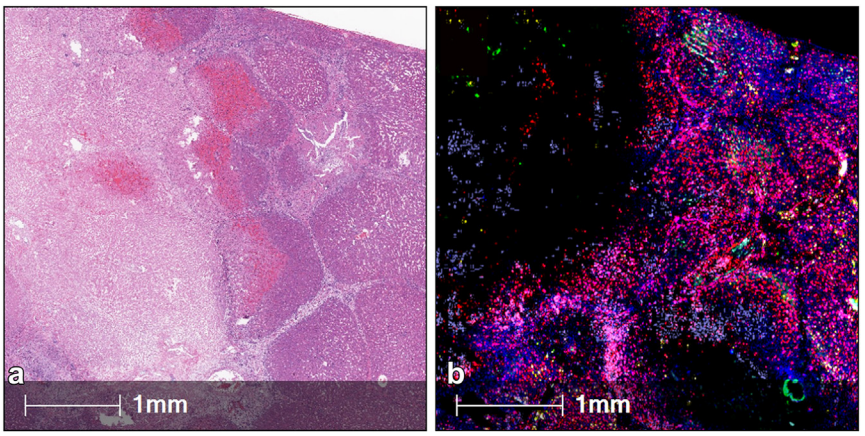

Figure 2. (a) Hematoxylin and eosin stain photomicrograph showed extensive area of necrosis on the left and hemorrhage at the margin. **(b)** Multiplex immunofluorescence image of adjacent section showed infiltration of large numbers of immune cells (CD45+, red). Abundant T cells (CD3+, magenta) were present among the immune infiltrate.

example using a solution of dichloroacetyl chloride in ethiodized oil, which was delivered in vivo in swine liver, into a lobar artery. Figure 2a is a standard hematoxylin and eosin section of tissue from 1 such experiment. Extensive coagulative necrosis can be appreciated on the left of the section with less severely affected liver on the right. Figure 2b is a multiplex immunofluorescence image of a matched adjacent section showing a brisk immune response, which includes T cells. Importantly, additional antibodies (FOXP3 and CD4) showed that very few T regulatory cells were present in the immune response. This near absence of regulatory cells bodes well for further development of thermoembolization in general and for potential synergies with immunotherapies. The dark area on the upper left side in Figure 2b corresponding to the necrotic zone in Figure 2a indicates that the antibodies failed to bind to any appreciable extent in this region. A composite multiplex image is shown in Figure 3. This implied that the antibodies no longer had a high affinity for their respective epitopes. Furthermore, in the same area, there is very little blue fluorescence from 4′,6-diamidino-2-phenylindole. This is a common fluorescent dye that functions as a fiducial marker or map in microscopy because it binds very reliably by intercalating within double-stranded DNA, illuminating cell nuclei. The most

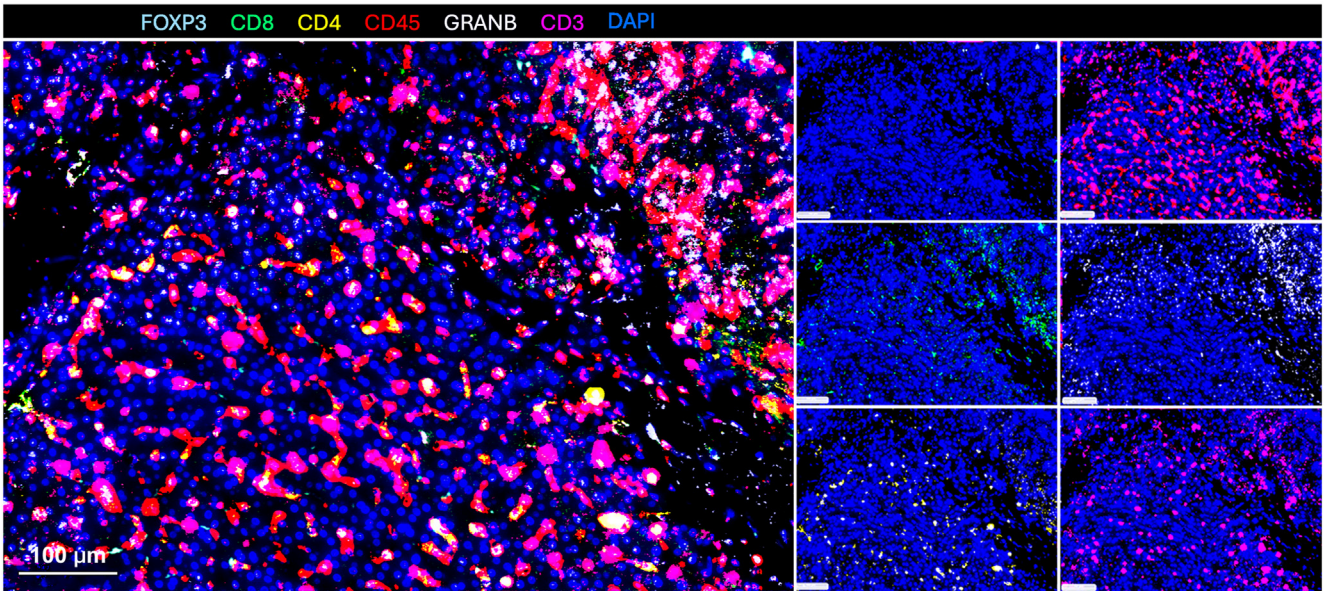

Figure 3. Multiplex Immunofluorescence. The composite image with individual channels below, showing high numbers of CD3+ T cells amongst the CD45+ immune population. T cells were predominantly CD4+ helper t cells, with moderate numbers of CD8+ cytotoxic T cells expressing granzyme b. CD3-CD45+granzyme b+ NK cells were also present. Regulatory T cells (FOXP3+CD3+CD4+) were rare to absent.

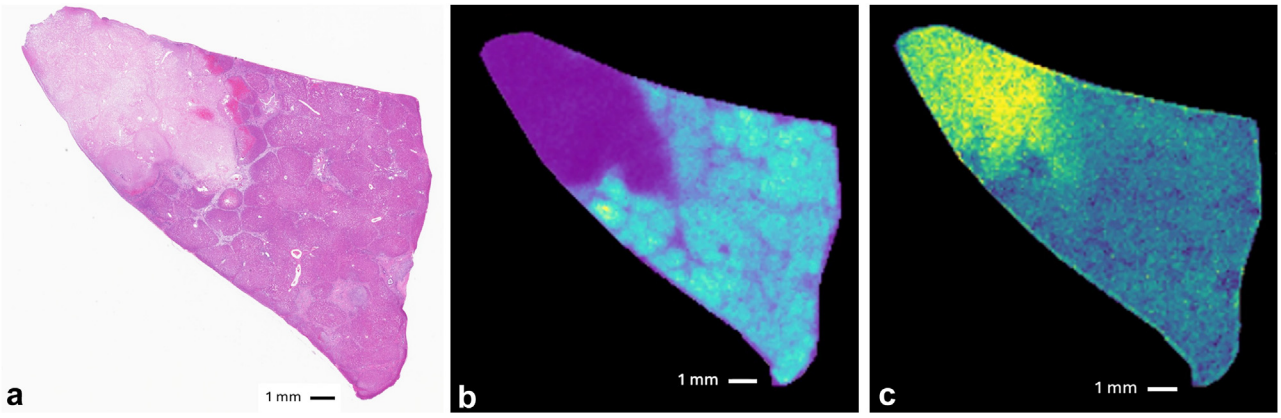

Figure 4. (a) Full section histologic section H&E stain after thermoembolization. Low power image corresponding to Fig. 2a. Confluent coagulative necrosis was extensive with a relatively sharp margin. Hemorrhage and inflammation at the margin were also evident. **(b)** Mass spectrometry image of a glycan tentatively assigned Hex8HexNAc2 – at 1743.5859 m/z ± 15 ppm. Note absence of signal (purple) in upper left region correlating with necrosis. **(c)** Mass spectrometry image of new signal at 1706.7711 m/z ± 15 ppm. Note increased signal (yellow) in upper left region, correlating with necrosis and not present in nearby unaffected tissue.

likely explanations for these findings are that the previously recognizable epitopes for the antibodies had undergone substantial changes as a result of the reaction(s) and that the local conditions were sufficiently extreme as to permanently disrupt DNA double-stranded structure.

This is all quite exciting in and of itself and an area of active research. However, a key data point was missing despite the authors' efforts. The authors could not readily identify the product expected from reaction with water (ie, hydrolysis). What follows is an almost retrograde path of discovery, leading to a new concept of modifying rather than killing cells. Indeed, it was much in the spirit of what the venerable biochemist and author Isaac Asimov once said, "The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny….'"

The absence of the expected product suggested that the authors' original hypothesis was incorrect. Because the data pointed away from water, the authors were forced to consider an alternative, unexpected reaction pathway. Contrary to the expectations of multiple chemists who considered the question, water was evidently not the primary reactant after all. In fact, it may not have participated to any meaningful extent. That left only tissue, which, from a chemist's perspective, is a complex array of amino acid side chains, lipids, nucleic acids, and glycans. This is depicted as the lower, unexpected pathway in **Figure 1**. The amine, sulfhydryl, or hydroxyl groups from any or all of these could serve as reaction partners. Shedding some light on this, molecular ion images from mass spectrometry imaging in **Figure 4b** and **c** offer a further glimpse of the situation. These pseudocolor images are of an adjacent full section as in **Figure 2a**. The full-section hematoxylin and eosin is shown for reference in **Figure 4a**. The geographic patterns for each molecular ion are distinct in each case and are correlated very well with the areas of coagulative necrosis. A detailed explanation of mass spectrometry imaging is beyond the scope of this discussion, but the important points are that in 1 case, a compound is *missing* in the damaged area (**Fig 4b**, purple region) and in the other, a *new* compound is present in the damaged area (**Fig 4c**, yellow region). The exact structure of these compounds remains to be determined, but taken together with the immunofluorescence results, the data provide strong evidence that *delivery of the reagent induced fundamental chemical changes in the tissue*.

CONCEPT

Having once considered the idea that controlled, covalent chemistry with locoregional precision might indeed be possible, the authors expanded their thinking beyond thermoembolization and cell death. The authors now began to consider the far broader chemical biology space of nondestructive/noncytotoxic reactions that would be much larger than the usual context of transarterial chemoembolization or even cancer. Many less reactive functional groups or warheads are available that would not kill cells, implying that locoregional chemistry to modify cells while preserving viability should be feasible. Of special interest for the current discussion is the proteome. In particular, the question arose as to how localized, image-guided chemistry could play an important role advancing knowledge in proteomics and in exploring new therapeutic opportunities.

A natural division exists between how and why for image-guided in vivo proteomics. The "why" is more easily addressed because proteins are critical in the structure, function, and signaling process of all cells. Protein production, modification, and turnover are a highly dynamic and context-sensitive process yet are difficult to study. The vast majority of protein studies are performed on cell lysates or purified, isolated proteins. Of those studies performed in vivo (a term used very broadly in this context it must be noted), nearly all are performed using cells grown in culture. This is a very artificial environment compared with the same cells in the living body of a human or animal. Thus, a major driver for the authors' interest is to more accurately profile which proteins are present in the native context and in what states of modification, not merely to characterize proteins isolated in the static conditions inherent to cell lysis.

With respect to how, the warhead or reactive functional group would serve as an anchor on a protein (**Fig 1**, unexpected path). Warheads can be chosen to impart chemical selectivity for specific amino acid residues on the solvent-accessible surface of a protein in addition to the spatial and temporal selectivity that image-guided methods provide. By incorporating an appropriate click chemistry handle elsewhere on a molecule, labeled protein sites could then be exploited for sample enrichment and proteomic analysis, imaging for spatial analysis through fluorescence or mass spectrometry, or all of the aforementioned.

RELEVANCE

The ability to perform localized chemistry using image guidance is anticipated to provide several benefits. In research, identifying proteins in their natural context will be a powerful new tool in drug discovery. It is likely that new protein targets and cell signaling pathways will be found for the simple reason that they were not present in vitro. Antibody-free local pretargeting with image guidance using click chemistry may enable new ways to deliver higher local drug concentrations with fewer off-target effects. Local alterations (also known as cell surface engineering) could create new possibilities not only in signaling and drug delivery but also in cell adhesion and trafficking. An obvious example would be modulating immune recognition in transplant organs, with the goal of decreasing side effects through decreased dosage or even potentially eliminating antirejection drugs. In oncology, protective masking of the vascular bed in organs known to be at risk such as the kidney could mean fewer side effects of antineoplastic and immune therapies. Conversely, enhanced immune recognition within an organ could mean more effective localization and activation of immune responses. Another may be artificially inducible nanoparticle enrichment. This would help address a major issue in therapeutic nanoparticles, namely, the efficiency problem. The rapid clearance of nanoparticles by the liver and spleen is well known, and local enrichment strategies may contribute to better delivery.

A novel advancement in this area is the in vivo mapping of changes in protein structures and interactions in response to the immune system (21). By introducing chemical probes with suitable handles directly into animals using embolization and intra-arterial drug infusion, researchers can capture

dynamic alterations at the molecular level. This innovative approach allows for the real-time observation of protein modifications and interactions in their native biological context, bypassing the limitations of traditional in vitro systems. By tracking immune-induced changes in protein dynamics, it will be possible to identify new targets for immune modulation and therapeutic intervention. This will offer deeper insights into immune responses and disease progression. As this technique advances, it could revolutionize how individuals study immune-related diseases, enabling more precise and personalized therapies. It is likely that additional research opportunities and clinical use scenarios will be revealed as this area continues to be developed.

TRANSLATION

Discovery research using in vivo chemistry, particularly in proteomics, will fall within the purview of academic centers and likely be limited to a handful given the specialized nature of the analytical techniques. Although the potential remains to be fully defined, as one understands better how to locally alter biology, the ramifications could be quite far-reaching. Clinical translation for therapeutic purposes, in contrast, is certainly foreseeable in the near future. Delivering new reagents in various formulations is well within reach using current catheter- and needle-based techniques. Projected barriers to advancement will instead likely be similar to those for any new drug testing, including assessing safety, effectiveness, metabolism, and off-target effects. In vivo proteomics offers the potential to revolutionize the way individuals understand diseases, develop treatments, and personalize therapies (22). By capturing protein changes in their natural environment, this approach will enable a more accurate, dynamic, and context-specific approach to biomedical research with the potential for significant advancements in diagnostics, therapeutics, and drug development. As the technology matures, it could play a key role in transforming the landscape of personalized medicine and improving patient outcomes on a global scale.

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