AOP Title

Protein Alkylation leading to Liver Fibrosis
Short name: Protein Alkylation to Liver Fibrosis

Authors

Brigitte Landesmann
Systems Toxicology Unit and EURL ECVAM, Institute for Health and Consumer Protection
European Commission Joint Research Centre,
Brigitte.LANDESMANN (at) ec.europa.eu

Status

Under development: Do not distribute or cite.

OECD Project 1.14: The Adverse Outcome Pathways from protein alkylation to liver fibrosis.

This AOP page was last modified on 2/20/2015.

Abstract

Hepatotoxicity in general is of special interest for human health risk assessment. Liver fibrosis in particular is an important human health issue associated with chemical exposure and predictive assays are lacking; it is a typical result of chronic or repeated-dose toxic injury and one of the considered endpoints for regulatory purposes. It is a long-term process in which inflammation, tissue destruction, and repair occur simultaneously, together with sustained production of growth factors and fibrogenic cytokines due to a complex interplay between various hepatic cell types, various receptors and signaling pathways which lead to an imbalance between the deposition and degradation of extracellular matrix (ECM) and a change of ECM composition. Due to this complex situation an adequate cell model is not available and an in vitro evaluation of fibrogenic potential is therefore not feasible. A sufficiently detailed description of the AOP to liver fibrosis might support chemical risk assessment by indicating early (upstream) markers for downstream events and facilitate a testing strategy without the need for a sophisticated cell model.

This AOP describes the linkage between hepatic injury caused by protein alkylation and the formation of liver fibrosis. The MIE is protein alkylation, leading to structural and functional cell injury and further to cell death, the first KE. Apoptotic hepatocytes undergo genomic DNA fragmentation and formation of apoptotic bodies. Upon engulfment of apoptotic bodies Kupffer cells (KCs) are activated, the next KE along the pathway. Activated KCs are the main source of TGF-β1, the most potent profibrogenic cytokine. TGF-β1 expression therefore is considered a KE that causes the next KE, hepatic stellate cell (HSCs) activation, meaning the transdifferentiation from a quiescent vitamin A–storing cell to a proliferative and contractile myofibroblast, the central effector in hepatic fibrosis. Activated HSCs cause progressive collagen accumulation, which together with changes in ECM composition signifies the KE on tissue level. Collagen bands progress further to bridging fibrosis, finally affecting the whole organ (the adverse outcome on organ level) and eventually leading to cirrhosis. Fibrous bands may disrupt normal blood flow, leading to portal hypertension and extensive scarring, which is the setting for unregulated growth and neoplasia. The inflammatory response plays an important role in driving fibrogenesis, since persistent inflammation precedes fibrosis. Inflammatory signaling stems from injured hepatocytes, activated KCs and HSCs. Inflammatory and fibrogenic cells stimulate each other in amplifying fibrosis. Chemokines and their receptors provoke further fibrogenesis, as well as interacting with inflammatory cells to modify the immune response during injury. Oxidative stress, as well, plays a crucial role in liver fibrogenesis by inducing hepatocyte apoptosis, activation of KCs and HSCs and fuelling inflammation. ROS contributing to oxidative stress are generated by hepatocytes, KCs, HSCs and inflammatory cells.

This purely qualitative AOP description is plausible, the scientific data supporting the AOP are logic, coherent and consistent and there is temporal agreement between the individual KEs. Quantitative data on dose-response-relationships and
temporal sequences between key events are still lacking; the provision of quantitative data will strengthen the weight of
evidence and make the AOP applicable for chemical risk assessment purposes.

Summary of the AOP

Molecular Initiating Event

**Protein, Alkylation**

**Short name:** Protein, Alkylation

How this Key Event works

**Level of Biological Organization**

Molecular

Covalent protein alkylation by reactive electrophiles was identified as a key triggering event in chemical toxicity over 40 years ago and these reactions remain a major cause of chemical-induced toxicity. Protein alkylation disturbs the cellular redox balance through interaction with glutathione, which leads to disruption of multiple biochemical pathways in exposed cells and is associated with mitochondrial dysfunction which in turn can trigger the death of exposed cells via either apoptosis and/or necrosis. Interestingly, some chemical molecules produce significant protein covalent binding without causing toxicity, which suggests that only a critical subset of protein alkylation events contributes to injury. The study by Codreanu et al. (2014) describes an inventory of electrophile-mediated protein damage in intact cells and suggests that non-toxic covalent binding may largely be survivable damage to cytoskeletal components, whereas toxic covalent binding produces lethal injury by targeting protein synthesis and catabolism and possibly mitochondrial electron transport. [1] Alkylating agents may also substitute alkyl groups for hydrogen atoms on DNA, resulting in the formation of cross links within the DNA chain and thereby resulting in cytotoxic, mutagenic, and carcinogenic effects. [2] [3] [4]

Introduction

Alkylation is the transfer of an alkyl group from one molecule to another. The alkyl group may be transferred as an alkyl carbocation, a free radical, a carbanion or a carbene (or their equivalents). [5] Protein alkylation is the addition of an alkyl group to a protein amino acid. An alkyl group is any group derived from an alkane by removal of one hydrogen atom. Alkylating agents are highly reactive chemicals that introduce alkyl radicals into biologically active molecules and thereby prevent their proper functioning. Many are used as antineoplastic agents, but most are very toxic, with carcinogenic, mutagenic, teratogenic, and immunosuppressant actions. Alkylating agents are classified according to their nucleophilic or electrophilic character. Nucleophilic alkylating agents deliver the equivalent of an alkyl anion (carbanion). These compounds typically can add to an electron-deficient carbon atom such as at a carbonyl group. Electrophilic alkylating agents deliver the equivalent of an alkyl cation. Alkyl halides can also react directly with amines to form C-N bonds; the same holds true for other nucleophiles such as alcohols, carboxylic acids, thiols, etc. Electrophilic, soluble alkylating agents are often very toxic, due to their ability to alkylate DNA. Alkylation with only one carbon is termed methylation. [6] [7]

How it is Measured or Detected

**HPLC-ESI-MS/MS analyse**

High Performance Liquid Chromatography – electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) is the most popular MS technique. It combines the separation ability of HPLC along with the sensitivity and specificity of detection from MS. One of the advantages of HPLC-MS is that it allows samples to be rapidly desalted online, so no sample preparation is required unlike samples for GC-MS. Electrospray ionisation can produce singly or multiply charged ions. Typically high molecular weight compounds have multiple charges i.e. peptides and proteins. This technique is particularly suited to analysing polar molecules of mass <2000Da and requires no prior derivatisation in most applications. [8] [9] [10]

**microsomal dealkylation assay** [11]
**Evidence Supporting Taxonomic Applicability**

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td></td>
<td>NCBI</td>
</tr>
</tbody>
</table>

**Evidence for Chemical Initiation of this Molecular Initiating Event**

Protein alkylation is a broad, non-specific MIE. Covalent protein alkylation is a feature of many hepatotoxic drugs but the overall extent of binding does not adequately distinguish toxic from non-toxic binding. \[^{[12]}\] Interestingly, some chemicals significantly alkylate proteins without causing toxicity, which suggests that only alkylation of a specific protein subset critical subset contributes to injury. Indeed, Codreanu presented an inventory of proteins affected by electrophile-mediated alkylation in intact cells and suggested that non-toxic covalent binding largely affects cytoskeletal protein components, whereas toxic covalent binding induces lethal injury by targeting factors involved in protein synthesis and catabolism and possibly mitochondrial electron transport \[^{[1]}\]. For this AOP it is unclear whether protein alkylation per se is sufficient to start the pathway or whether alkylation to specific proteins or families of proteins needs to be affected and whether various binding sites influence the further downstream process. The identification and specification of the targeted biomolecules is needed for the structural definition of chemical initiators and consequentially for profiling and categorising of chemicals related to the initiation of this AOP. Likewise it is necessary for the establishment of a distinct relationship with the next downstream event. Further it is unknown whether there is a threshold and if this threshold would refer to the number of alkylation of a single protein or of a threshold number of proteins. DNA alkylation (methylation) could play a role as well, but for the moment there is no sufficient data to substantiate this claim. Future studies could provide a better mechanistic basis for interpreting protein alkylation in chemical safety evaluation. The initial AOP case study was based on data of two prototypic fibrogenic chemicals, namely Carbon Tetrachloride (CCl4) and Allyl Alcohol. Further knowledge was gathered by using more known fibrogenic chemicals, namely Thioacetamide, Amiodarone, Methotrexate, Isoniazid, Dimethyl Nitrosamine, Ethanol, Retinol, Ethinyl Estradiol, and Chlorpromazine. These chemicals were selected as known inducers of liver fibrosis. An extensive literature search looking for information from in vivo repeated dose toxicity studies was performed and the gathered knowledge was used to confirm the sequence of mechanistic events leading to liver fibrosis. Further a structural analysis has been performed to assess structural similarities between these 11 fibrogenic chemicals and to identify potential common sub-structures in order to possibly "correlate" structure with toxicological potential. Structural similarities could not be found; the only commonality was that all - either the parent compound or active metabolites - were protein binders without any identifiable distinguishing features between fibrogenic and non fibrogenic hepatotoxicants, as well as non-hepatotoxic chemicals.

**References**

1. \[^{[10]}\] Codreanu et al., 2014, Alkylation damage by lipid electrophiles targets functional protein systems, Molecular & Cellular Proteomics 13.3, 849–859
2. \[^{[11]}\] Grattagliano et al., (2009), Biochemical mechanisms in drug-induced liver injury: certainties and doubts, World J Gastroenterol. 15(39): 4865-4876
6. The European Bioinformatics Institute [http://www.ebi.ac.uk/QuickGO/GTerm?id=GO:0008213](http://www.ebi.ac.uk/QuickGO/GTerm?id=GO:0008213)

References for chemical initiators:
The pathogenesis of drug- or toxin-induced cell injury usually involves the participation of toxic metabolites that either elicit an immune response or directly affect the biochemistry of the cell. Intracellular stress can lead to apoptotic or necrotic cell death, depending on the extent of mitochondrial involvement and the balance of factors that activate and inhibit the Bcl2 family of proteins and the caspases. Chemicals and their metabolites can undergo or promote a variety of chemical reactions, including covalent binding, depletion of reduced glutathione, or oxidative stress with consequent effects on proteins, lipids, and DNA. These chemical consequences can directly affect organelles such as mitochondria, cytoskeleton, endoplasmic reticulum, microtubules, or nucleus or indirectly influence these organelles through activation or inhibition of signaling kinases, transcription factors, and gene expression profiles. The outcome may be either triggering of the necrotic or apoptotic process or sensitization to the lethal action of cytokines of the immune system.

Two types of cell death can be distinguished by morphological features, although it is likely that these are two ends of a spectrum with possible intermediate forms. Apoptosis involves shrinkage, nuclear disassembly, and fragmentation of the cell into discrete bodies with intact plasma membranes. These are rapidly phagocytosed by neighbouring cells. An important feature of apoptosis is the requirement for adenosine triphosphate (ATP) to initiate the execution phase. In contrast, necrotic cell death is characterized by cell swelling and lysis. This is usually a consequence of profound loss of mitochondrial function and resultant ATP depletion, leading to loss of ion homeostasis, including volume regulation, and increased Ca2+; the latter activates a number of nonspecific hydrolases (i.e., proteases, nucleases, and phospholipases). A fundamental distinction between necrosis and apoptosis is the loss of plasma membrane integrity; this is integral to the former but not the latter. As a consequence, lytic release of cellular constituents promotes a local inflammatory reaction, whereas the rapid removal of apoptotic bodies minimizes such a reaction.

The distinction between the two modes of death is easily accomplished in vitro but not in vivo. Thus, although claims that certain drugs induce apoptosis have been made, these are relatively unconvincing. DNA fragmentation can occur in
necrosis, leading to positive TUNEL staining of liver sections. Conversely, when apoptosis is massive, it can exceed the capacity for rapid phagocytosis, resulting in the eventual appearance of secondary necrosis.

Two alternative pathways - either extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) - lead to apoptotic cell death. The initiation of cell death begins either at the plasma membrane with the binding of TNF or FasL to their cognate receptors or within the cell. The latter is due to the occurrence of intracellular stress in the form of biochemical events such as oxidative stress, redox changes, covalent binding, lipid peroxidation, and consequent functional effects on mitochondria, endoplasmic reticulum, microtubules, cytoskeleton, or DNA. Intracellular stress either directly affects mitochondria or can lead to effects on other organelles, which then send signals to the mitochondria to recruit participation in the death process. [1] [2]

How it is Measured or Detected

Necrosis: LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged. To detect the leakage of LDH into cell culture medium, a tetrazolium salt is used in this assay. In the first step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is converted to a colored formazan product using newly synthesized NADH in the presence of an electron acceptor. The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to enumerate the percentage of necrotic cells in a sample. [3] Apoptosis: TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase or TdT, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage. [4]

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>human and other cells in culture</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

Cell death is an universal event occurring in cells of any species.

References

1. ↑ Malhi, H. et al., (2010), Hepatocyte death: a clear and present danger. Physiol Rev. 90, 1165-1194

Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=21509

Hepatic macrophages (Kupffer Cells), Activation and Recruitment

Short name: Hepatic macrophages (Kupffer Cells), Activation and Recruitment

How this Key Event works

<table>
<thead>
<tr>
<th>Level of Biological Organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular</td>
</tr>
</tbody>
</table>

Hepatic macrophages (Kupffer Cells) constitute 80%-90% of the tissue macrophages in the reticuloendothelial system and account for approximately 15% of the total liver cell population [1]. Activated KCs are involved in the pathogenesis of chemical- or toxin-induced liver injury through the release of inflammatory mediators including cytokines, chemokines, lysosomal and proteolytic enzymes and are a main source of TGF-β1 (transforming growth factor-beta 1, the most potent profibrogenic cytokine). In addition latent TGF-β1 can be activated by KC-secreted matrix metalloproteinase 9 (MMP-9). [2] [3] through the release of biologically active substances that promote the pathogenic process. Activated KCs also release ROS like superoxide generated by NOX (NADPH oxidase), thus contributing to oxidative stress. Oxidative stress also activates a
variety of transcription factors like NF-κB, PPAR-γ leading to an increased gene expression for the production of growth factors, inflammatory cytokines and chemokines. KCs express TNF-α (Tumor Necrosis Factor-alpha), IL-1 (Interleukin-1) and MCP-1 (monocyte-chemoattractant protein-1), all being mitogens and chemoattractants for HSCs and induce the expression of PDGF receptors on HSCs which enhances cell proliferation. Expressed TNF-α, TRAIL (TNF-related apoptosis-inducing ligand), and FasL (Fas Ligand) are not only pro-inflammatory active but also capable of inducing death receptor-mediated apoptosis in hepatocytes. Under conditions of oxidative stress macrophages are further activated which leads to a more enhanced inflammatory response that again further activates KCs though cytokines (Interferon gamma (IFNγ), granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α), bacterial lipopolysaccharides, extracellular matrix proteins, and other chemical mediators. [4] [5] Expressed TNF-α, TRAIL (TNF-related apoptosis-inducing ligand), and FasL (Fas Ligand) are not only pro-inflammatory active but also capable of inducing death receptor-mediated apoptosis in hepatocytes. [6] [7] [8] Besides Kupffer cells, the resident hepatic macrophages, infiltrating bone marrow-derived macrophages, originating from circulating monocytes are recruited to the injured liver via chemokine signals. Kupffer cells appear essential for sensing tissue injury and initiating inflammatory responses, while infiltrating Ly-6C+ monocyte-derived macrophages are linked to chronic inflammation and fibrogenesis. The profibrotic functions of Kupffer cells (HSC activation via paracrine mechanisms) during chronic hepatic injury remain functionally relevant, even if the infiltration of additional inflammatory monocytes is blocked via pharmacological inhibition of the chemokine CCL2 [9] [10] KC activation and macrophage recruitment are two separate events and both are necessary for fibrogenesis, but as they occur in parallel, they can be summarised as one key event. Probably there is a threshold of KC activation and release above which liver damage is induced. Pre-treatment with gadolinium chloride (GdCl), which inhibits Kupffer cell function, reduced both hepatocyte and sinusoidal epithelial cell injury, as well as decreased the numbers of macrophages appearing in hepatic lesions and inhibited TGF-β1 mRNA expression in macrophages. Experimental inhibition of KC function or depletion of KCs appeared to protect against chemical-induced liver injury.[11]

How it is Measured or Detected

Kupffer cell activation can be measured by means of expressed cytokines, e.g. tissue levels of TNF-α [12], IL-6 expression, measured by immunoassays or Elisa (offered by various companies), soluble CD163 [13] [14] or increase in expression of Kupffer cell marker genes such as Lyz, Gzmb, and Il1b, (Genome U34A Array, Affymetrix); [15].

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>human and other cells in culture</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

Humans (all references)

Rats: [3]

Mice: [16]

References

1. ↑ Bouwens et al., 1986, Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver, Hepatology; 6: 718-722
2. ↑ Winwood et al., 1993, Kupffer cells: their activation and role in animal models of liver injury and human liver disease, Semin Liver Dis; 13: 50-59
8. ↑ Roberts et al., 2007, Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis, Toxicol Sci.; 96(1): 2-15
9. ↑ Baeck et al., 2012, Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage
infiltration and steatohepatitis in chronic hepatic injury. Gut;61:416–426

10. † Tacke and Zimmermann, 2014, Macrophage heterogeneity in liver injury and fibrosis, J Hepatol.;60(5):1090-6

11. † Ide et al., 2005, Effects of gadolinium chloride (GdCl(3)) on the appearance of macrophage populations and fibrogenesis in thioacetamide-induced rat hepatic lesions J. Comp. Path., Vol. 133, 92–102

12. † Vajdova et al., ischemic preconditioning and intermittent clamping improve murine hepatic microcirculation and Kupffer cell function after ischemic injury. Liver Transpl 2004;10:520–528


15. † Takahara et al, Gene expression profiles of hepatic cell-type specific marker genes in progression of liver fibrosis. World J Gastroenterol 2006 October 28; 12(40): 6473-6499

16. † Dalton et al., 2009, Carbon tetrachloride-induced liver damage in asialoglycoprotein receptor-deficient mice, Biochem Pharmacol. 1;77(7):1283-1290

Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20876

**TGFbeta1 expression, Up Regulation**

**Short name:** TGFbeta1 expression, Up Regulation

**How this Key Event works**

### Level of Biological Organization

**Cellular**

Transforming growth factor beta 1 or TGF-β1 is a polypeptide member of the transforming growth factor beta superfamily of cytokines. TGF-β is synthesized as a non-active pro-form, forms a complex with two latent associated proteins latency-associated protein (LAP) and latent TGF-β binding protein (LTBP) and undergoes protilithic cleavage by the endopeptidase furin to generate the mature TGF-β dimer. Three TGF-β isoforms (β1, β2 and β3) have been identified, but only TGF-β1 was linked to liver fibrogenesis and is the most potent fibrogenic factor for HSCs. It plays a central role in fibrogenesis, mediating a cross-talk between parenchymal, inflammatory and collagen expressing cells. TGF-β1 is released by activated Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and platelets; in the further course of events also activated HSCs express TGF-β1. Hepatocytes do not produce TGF-β1 but are implicated in intracellular activation of latent TGF-β1. [1] [2] [3] [4] [5] TGF-β1 induces its own mRNA to sustain high levels in local sites of liver injury. The effects of TGF-β1 are classically mediated by intracellular signaling via Smad proteins. Smads 2 and 3 are stimulatory whereas Smad 7 is inhibitory. [6] [7] [8] Smad1/5/8, MAP kinase (mitogen-activated protein) and PI3 kinase are further signaling pathways in different cell types for TGF-β1 effects. TGF-β1 activates HSCs, stimulates ECM (extracellular matrix) synthesis and suppresses ECM degradation. TGF-β1 activates HSCs, stimulates extracellular matrix (ECM) synthesis and suppresses ECM degradation. It stimulates collagen transcription in HSCs and expression of connective tissue growth factor (CTGF), a profibrogenic peptide that stimulates the synthesis of collagen type I and fibronectin and induces the expression of TIMP-1 (tissue inhibitor of metalloproteinases -1), an inhibitor of the collagen cleaving enzymes MMP-8 and MMP-13. TGF-β1 increases the α1(I) collagen mRNA half-life, mediated by increasing stability of α1(I) collagen mRNA through mitogen-activated protein kinases (MAPK). TGF-β1 further recruits inflammatory cells, portal fibroblasts and circulating myofibroblasts to injured liver and triggers apoptosis of hepatocytes. [2] [9] [3] [6] [10] TGF-β1 is the most established mediator and regulator of epithelial-mesenchymal-transition (EMT) which further contributes to the production of extracellular matrix. It has been shown that TGF-β1 mediates EMT by inducing snail-1 transcription factor and tyrosine phosphorylation of Smad2/3 with subsequent recruitment of Smad4. [11] [12] [3] [14] [15] [10] [9] [16] [17] [3] [4] [6] [18] [5] Liver injury by a variety of means results in a rapid induction of TGF-β synthesis consistent with a ubiquitous role for TGF-β in wound healing. During fibrogenesis, tissue and blood levels of active TGF-β are elevated and overexpression of TGF-β1 in transgenic mice can induce fibrosis. Additionally, experimental fibrosis can be inhibited by anti-TGF-β treatments with neutralizing antibodies or soluble TGF-β receptors [19] [20] [21] [22]

**How it is Measured or Detected**

There are several assays for TGB-β1 measurement available.

**e.g.** Human TGF-β1 ELISA Kit (Sigma Aldrich) The Human TGF-β 1 ELISA (Enzyme –Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TGF-β1 in serum, plasma, cell culture supernatants, and urine. This assay employs an antibody specific for human TGF-β1 coated on a 96-well plate. Standards and samples are pipetted into the wells and TGF-β1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TGF-β1 antibody is added. After washing away unbound biotinylated antibody, HRP- conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB
substrate solution is added to the wells and colour develops in proportion to the amount of TGF-β1 bound. The StopSolution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm [23]

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>human and other cells in culture</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

Humans

Rats: [24]

Mice: [25]

References

2. ↑ 2.0 2.1 Kisseleva and Brenner 2007,Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis, Journal of Gastroenterology and Hepatology 22, Suppl. 1; S73-S78
4. ↑ 4.0 4.1 Poli 2000, Molecular Aspects of Medicine 21, 49 - 98
5. ↑ 5.0 5.1 5.2 Liu et al. 2006, Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. Liver Int. 26, 8-22
6. ↑ 6.0 6.1 6.2 Parsons 2007, Molecular mechanisms of hepatic fibrogenesis. J Gastroenterol Hepatol. 22, S79-S84
10. ↑ 10.0 10.1 Gressner et al. 2002, Roles of TGF-β in hepatic fibrosis. Front Biosci. 1,793-807
16. ↑ Li, 2008, Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies, J Gastroenterol; 43:419-428
19. ↑ Qi et al., 1999 Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat, Proc Natl Acad Sci U S A 96, 2345-2349
24. ↑ Luckey et al, 2001, Activation of Kupffer cells during the course of carbon tetrachloride-induced liver injury and fibrosis in rats Experimental and Molecular Pathology 71, 226-240
25. ↑ Nan et al., 2013, Activation of peroxisome proliferator activated receptor alpha ameliorates ethanol mediated liver fibrosis in mice. Lipids in Health and Disease, 12 :11
Stellate cells, Activation
Short name: Stellate cells, Activation

How this Key Event works

Level of Biological Organization

Cellular

Stellate cell activation means a transdifferentiation from a quiescent vitamin A–storing cell to a proliferative and contractile myofibroblast and is the dominant event in liver fibrosis. Multiple cells and cytokines play a part in the regulation of HSC activation that consists of discrete phenotype responses, mainly proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis and retinoid loss.

HSCs undergo activation through a two-phase process. The first step, the initiation phase is triggered by injured hepatocytes, ROS and paracrine stimulation from neighbouring cell types (KCs, LSECs, and platelets) and make HSCs sensitized to activation by up-regulating various receptors. The perpetuation phase refers to the maintenance of HSC activation, which is a dynamic process including the secretion of autocrine and paracrine growth factors (such as TGF-β1), chemokines, and the up-regulation of collagen synthesis (mainly type I collagen). In response to growth factors (including PDGF and VEGF) HSCs proliferate. Increased contractility (Endothelin-1 and NO are the key opposing counter-regulators that control HSC contractility, in addition to angiotensinogen II, and others) leads to increased portal resistance. Driven by chemoattractants their accumulation in areas of injury is enhanced. TGF-β1 synthesis promotes activation of neighbouring quiescent hepatic stellate cells, whereas the release of HGF (hepatoctye growth factor) stimulates regeneration of adjacent hepatocytes. The release of chemoattractants (monocyte chemoattractant protein-1, MCP-1 and colony-stimulating factors, CSFs) amplifies inflammation. [1], [2] [3] [4], [5] [6] [7] Activated HSCs (myofibroblasts) are the primary collagen producing cell, the key cellular mediators of fibrosis and a nexus for converging inflammatory pathways leading to fibrosis. Experimental inhibition of stellate cell activation prevents fibrosis. [8] [9]

How it is Measured or Detected

alpha-smooth muscle actin (α-SMA) is a well-known marker of hepatic stellate cells activation Anti-alpha smooth muscle Actin [1A4] monoclonal antibody reacts with the alpha smooth muscle isoform of actin.

Gene expression profiling confirmed early changes for known genes related to HSC activation such as alpha smooth muscle actin (Acta2), lysyl oxidase (Lox) and collagen, type I, alpha 1 (Col1a1). Insulin-like growth factor binding protein 3 (Igfbp3) was identified as a gene strongly affected and as marker for culture-activated HSCs and plays a role in HSC migration. [10] [11]

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>human and other cells in culture</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

Humans, Rats, Mice, Pigs [3]

References

8. Li JT et al, Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies, J Gastroenterol 2008; 43:419-428


Collagen, Accumulation
Short name: Collagen, Accumulation

How this Key Event works

<table>
<thead>
<tr>
<th>Level of Biological Organization</th>
<th>Tissue</th>
</tr>
</thead>
</table>

Irrespective of upstream events that trigger and entertain fibrosis, the final product of myofibroblast cellular activity is the massive deposition of collagen which results in fibrosis. The overall amount of collagen deposited by fibroblasts is a regulated balance between collagen synthesis and collagen catabolism. HSCs generate fibrosis not only by increasing cell number, but also by increasing matrix production per cell. The basement membrane-like matrix is normally comprised of collagens IV and VI, which is progressively replaced by collagens I and III and cellular fibronectin during fibrogenesis. Although multiple ECM components are dramatically upregulated in hepatic fibrosis, type I collagen is the most abundant protein. Synthesis of type I collagen is initiated by expression of the col1a1 and col1a2 genes, giving rise to α 1(I) and α 2(I) procollagen mRNAs, respectively. Levels of these gene products can be regulated at both the transcriptional and post-transcriptional level. Despite being located on different chromosomes, expression of these two genes are coordinately regulated in a tissue-specific manner giving rise to a 1(I) and a 2(I) procollagen mRNA products, respectively. Levels these changes in ECM composition initiate several positive feedback pathways that further amplify fibrosis.

Increasing matrix stiffness is a stimulus for HSC activation. Matrix-provoked signals link to other growth factor receptors through integrin-linked kinase and transduce via membrane-bound guanosine triphosphate binding proteins, in particular Rho67 and Rac, signals to the actin cytoskeleton that promote migration and contraction. Activation of cellular matrix MMPs leads to release of growth factors from matrix-bound reservoirs in the extracellular space that further stimulate cellular growth and fibrogenesis. Besides the transition of quiescent HSCs into activated HSCs and then further into contractile myofibroblasts, other cells may transdifferentiate into fibrogenic myofibroblasts in liver injury. Additional sources of ECM include bone marrow (which probably gives rise to circulating fibrocytes), portal fibroblasts, EMT (epithelial–mesenchymal cell transition) from hepatocytes and cholangiocytes.

How it is Measured or Detected

Determiner of the amount of collagen produced in vitro can be done in a variety of ways ranging from simple colorimetric assays to elaborate chromatographic procedures using radioactive and non-radioactive material. What most of these procedures have in common is the need to destroy the cell layer to obtain solubilized collagen from the pericellular matrix. Rishikof et al describe several methods to assess the in vitro production of type I collagen: Western immunoblotting of intact alpha1(I) collagen using antibodies directed to alpha1(I) collagen amino and carboxyl propeptides, the measurement of alpha1(I) collagen mRNA levels using real-time polymerase chain reaction and methods to determine the transcriptional regulation of alpha1(I) collagen using a nuclear run-on assay.

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>
Mice: [4], [5], [6]

Rats: [7], [8], [9], [10]

References


Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20951

Oxidative Stress, Increase
Short name: Oxidative Stress, Increase

How this Key Event works

<table>
<thead>
<tr>
<th>Level of Biological Organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular</td>
</tr>
</tbody>
</table>

Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation. The term oxidative stress indicates the outcome of oxidative damage to biologically relevant macromolecules such as nucleic acids, proteins, lipids and carbohydrates. This occurs when oxidative stress-related molecules, generated in the extracellular environment or within the cell, exceed cellular antioxidant defenses. Major reactive oxygen species (ROS), such as hydrogen peroxide (H2O2) and superoxide anion, as well as 4-hydroxy- 2,3-nonenal (HNE) and related 4-hydroxy-2,3-alkenals (HAKs), major aldehydic end-products of lipid peroxidation, can act as potential mediators able to affect signal transduction pathways as well as the proliferative and functional response of target cells. H2O2 and superoxide anion may be also generated as molecular messengers within the cell as part of the cellular response to defined growth factors, cytokines and other mediators. The final consequence at tissue, cellular and molecular level is primarily affected by the steady state concentration of oxidative stress-related molecules. The main biological targets of free radicals are proteins, lipids and DNA.

Major consequences of reaction of ROS, HAKs and NO with biologically relevant macromolecules that can mediate pathophysiological effects:

ROS: DNA: oxidation, strand breaks, genotoxicity Proteins: oxidation, fragmentation, formation of carbonyls Lipids: lipid peroxidation and degradation
HAKs: DNA: adducts (low doses), strand breaks, genotoxicity (high doses) Proteins: adducts (Michael type reactions on Lys, Cys and His residues)

NO: DNA: oxidation, strand breaks Proteins: oxidation, nitrosation, nitration (nytrosylation of tyrosine) Lipids: lipid peroxidation and degradation

Continued oxidative stress can lead to chronic inflammation. Oxidative stress can activate a variety of transcription factors including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt, and Nrf2. Activation of these transcription factors can lead to the expression of over 500 different genes, including those for growth factors, inflammatory cytokines and chemokines, which can activate inflammatory pathways. [1] [2] [3]

**How it is Measured or Detected**

**measuring oxidative stress**

Agents for ROS detection are primarily fluorescence based, but recently luminescent based detections have been introduced. The biggest difficulty reported with much of the cellular ROS research has been with the lack of reporter agents specific for discrete molecules. ROS moieties by their nature are reactive with a number of different molecules; as such designing reporter agents has been difficult. With more specific chemistries, particularly for hydrogen peroxide, the specific mechanisms for regulation will be elucidated.

Reduced glutathione (GSH) is regenerated from its oxidized form (GSSH) by the action of an NADPH dependent reductase GSSH + NADPH + H+ à 2 GSH + NADP+ Due to the rapid nature of the reduction of GSSH relative to its synthesis or secretion, the ratio of GSH to GSSH is a good indicator of oxidative stress within cells. GSH and GSSH levels can be determined by HPLC, capillary electrophoresis, or biochemically in microplates. Several different assays have been designed to measure glutathione in samples. By using a luciferin derivative in conjunction with glutathione S-transferase enzyme the amount of GSH would be proportional to the luminescent signal generated when luciferase is added in a subsequent step. Total glutathione can be determined colorimetrically by reacting GSH with DTNB (Ellman’s reagent) in the presence of glutathione reductase. Glutathione reductase reduces GSSH to GSH, which then reacts with DTNB to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB), which absorbs at 412 nm.

Lipid peroxidation is one of the most widely used indicators of free radical formation, a key indicator of oxidative stress. Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA) reactive compounds such as malondialdehyde generated from the decomposition of lipid peroxidation products. While this method is controversial in that it is quite sensitive, but not necessarily specific to MDA, it remains the most widely used means to determine lipid peroxidation. This reaction, which takes place under acidic conditions at 90-100°C, results in an adduct that can be measured colorimetrically at 532 nm or by fluorescence using a 530 nm excitation wavelength and a 550 nm emission wavelength. A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies. The formation of F2-like prostanoid derivatives of arachidonic acid, termed F2-isoprostanes (IsoP) has been shown to be specific for lipid peroxidation. Unlike the TBA assay, measurement of IsoP appears to be specific to lipid peroxides, they are stable and are not produced by any enzymatic pathway making interpretation easier. There have been a number of commercial ELISA kits developed for IsoPs, but interfering agents in samples requires partial purification of samples prior to running the assay. The only reliable means for detection is through the use of GC/MS, which makes it expensive and limits throughput.

Superoxide detection is based on the interaction of superoxide with some other compound to create a measurable result. The reduction of ferricytochrome c to ferrocytochrome c has been used in a number of situations to assess the rate of superoxide formation. While not completely specific for superoxide this reaction can be monitored colorimetrically at 550 nm. Chemiluminescent reactions have been used for their potential increase in sensitivity over absorbance-based detection methods. The most widely used chemiluminescent substrate is Lucigenin, but this compound has a propensity for redox cycling, which has raised doubts about its use in determining quantitative rates of superoxide production. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical. These dyes are synthesized by reducing the iminium cation of the cyanine (Cy) dyes with sodium borohydride. While weakly fluorescent, upon oxidation their fluorescence intensity increases 100 fold. In addition to being fluorescent, oxidation also converts the molecule from being membrane permeable to an ionic impermeable moiety. The most characterized of these probes are Hydro-Cy3 and Hydro-Cy5.

Hydrogen peroxide (H2O2) is the most important ROS in regards to mitogenic stimulation or cell cycle regulation. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products. The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H2O2 form increasing amounts of fluorescent product.

Nitric Oxide The free radical nitric oxide ([NO]) is produced by a number of different cell types with a variety of biological functions. Regardless of the source or role, the free radical •NO has a very short half life (t½ = 4 seconds), reacting with...
several different molecules normally present to form either nitrate (NO3-) or nitrite (NO2-) A commonly used method for the indirect determination of •NO is the determination of its composition products nitrate and nitrite colorimetrically. This reaction requires that nitrate (NO3) first be reduced to nitrite (NO2), typically by the action of nitrate reductase. Subsequent determination of nitrite by a two step process provides information on the “total” of nitrate and nitrite. In the presence of hydrogen ions nitrite forms nitrous acid, which reacts with sulfanilamide to produce a diazonium ion. This then coupled to N-(1-napthyl) ethylenediamine to form the chromophore which absorbs at 543 nm. Nitrite only determinations can then be made in a parallel assay where the samples were not reduced prior to the colorimetric assay. Actual nitrate levels are then calculated by the subtraction of nitrite levels from the total. [4]

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>human and other cells in culture</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

References


Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20979

Chronic Inflammation, Increase
Short name: Chronic Inflammation, Increase

How this Key Event works

Level of Biological Organization

Tissue

Inflammation is part of the complex biological response of tissues to harmful stimuli, such as pathogens, damaged cells, or toxicants. The process of acute inflammation is initiated by cells already present in all tissues, as monocytes and macrophages, which produce nitric oxide and inflammatory cytokines.

Hepatic infiltration of neutrophils is an acute response to liver injury and hepatic stress. Neutrophils are activated by proinflammatory cytokines (cytokines represent a family of biologic response modifiers including interleukins, chemokines, interferons, growth factors, and leukocyte colony-stimulating factors), accumulate in the hepatic microvasculature and transmigrate into the hepatic parenchyma mediated by a chemokine gradient and orchestrated by adhesion molecules.

The acute inflammatory response requires constant stimulation to be sustained.

Histopathologic features of chronic inflammation include the predominance of macrophages and lymphocytes, proliferation of nurturing structurally heterogeneous and hyperpermeable small blood vessels, fibrosis, and necrosis. Activated macrophages and lymphocytes are interactive in releasing inflammatory mediators or cytokines that amplify immune reactivity. In chronic inflammation, the protracted inflammatory response is often accompanied simultaneously by tissue destruction and repair.

Inflammatory process induces oxidative stress and reduces cellular antioxidant capacity. Overproduced free radicals react with cell membrane fatty acids and proteins impairing their function permanently. Activated inflammatory cells (NADH/ NADPH oxidase systems in neutrophils, macrophages and Kupffer cells) represent a major source of oxidative stress-related molecules. Inflammatory cells secrete a large number of cytokines and chemokines. Reactive oxygen and nitrogen species are produced under the stimulus of pro-inflammatory cytokines in phagocytic and non-phagocytic cells through the
activation of protein-kinases signaling. For example, TNF-α enhances the formation of ROS by neutrophils and other cells, while interleukin-1-b, TNF-α and interferon-g stimulate the expression of inducible nitric oxide synthase in inflammatory and epithelial cells. [1] [2] [3] [4] [5] [6] [7]

**How it is Measured or Detected**

release of inflammatory cytokines like Interleukins, Interferons, TNF-α (measurement based on monoclonal antibodies, ELISA), gene expression analysis, mRNA levels;

**Evidence Supporting Taxonomic Applicability**

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

**References**


**Adverse Outcome**

Liver fibrosis, N/A

**Liver fibrosis, N/A**

**Short name:** Liver fibrosis, N/A

**How this Key Event works**

**Level of Biological Organization**

Organ

Liver fibrosis results from perpetuation of the normal wound healing response, as a result of repeated cycles of hepatocyte injury and repair and is a dynamic process, characterised by an excessive deposition of ECM (extracellular matrix) proteins including glycoproteins, collagens, and proteoglycans. It is usually secondary to hepatic injury and inflammation, and progresses at different rates depending on the aetiology of liver disease and is also influenced by environmental and genetic factors. If fibrosis continues, it disrupts the normal architecture of the liver, altering the normal function of the organ and ultimately leading to liver damage. Cirrhosis represents the final stage of fibrosis. It is characterised by fibrous septa which divide the parenchyma into regenerative nodules which leads to vascular modifications and portal hypertension with its complications of variceal bleeding, hepatic encephalopathy, ascites, and hepatorenal syndrome. In addition, this condition is largely associated with hepatocellular carcinoma with a further increase in the relative mortality rate. [1] [2]

**How it is Measured or Detected**

Liver biopsy has been used to assess the degree of hepatic inflammation and fibrosis. This is an invasive test with many possible complications and the potential for sampling error. Noninvasive tests are increasingly precise in identifying the amount of liver fibrosis. Standard liver tests are of limited value in assessing the degree of fibrosis. Direct serologic markers
of fibrosis include those associated with matrix deposition — e.g. procollagen type III amino-terminal peptide (P3NP), type I and IV collagens, laminin, hyaluronic acid, and chondrex. P3NP is the most widely studied marker of hepatic fibrosis. Other direct markers of fibrosis are those associated with matrix degradation, i.e., matrix metalloproteinases 2 and 3 (MMP-2, MMP-3) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1, TIMP-2). These tests are not commercially available, and the components are not readily available in most clinical laboratories. Some indirect markers that combine several parameters are available but not very reliable. Conventional imaging studies (ultrasonography and computed tomography) are not sensitive for fibrosis. Hepatic elastography, a method for estimating liver stiffness, is a recent development in the noninvasive measurement of hepatic fibrosis. Currently, elastography can be accomplished by ultrasound or magnetic resonance. Liver biopsy is still needed if laboratory testing and imaging studies are inconclusive. [3] [4]

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td>Strong</td>
<td>NCBI</td>
<td></td>
</tr>
</tbody>
</table>

Regulatory Examples Using This Adverse Outcome

From the OECD - GUIDANCE DOCUMENT ON DEVELOPING AND ASSESSING ADVERSE OUTCOME PATHWAYS - Series on Testing and Assessment 18: "...an adverse effect that is of regulatory interest (e.g. repeated dose liver fibrosis)"

References


Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20853

Scientific evidence supporting the linkages in the AOP

<table>
<thead>
<tr>
<th>Event</th>
<th>Description</th>
<th>Triggers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, Alkylation</td>
<td>Directly Leads to</td>
<td>Cell death, N/A</td>
</tr>
<tr>
<td>Cell death, N/A</td>
<td>Directly Leads to</td>
<td>Hepatic macrophages (Kupffer Cells), Activation</td>
</tr>
<tr>
<td>Cell death, N/A</td>
<td>Indirectly Leads to</td>
<td>Stellate cells, Activation</td>
</tr>
<tr>
<td>Hepatic macrophages (Kupffer Cells), Activation</td>
<td>Directly Leads to</td>
<td>TGFbeta1 expression, Up Regulation</td>
</tr>
<tr>
<td>TGFbeta1 expression, Up Regulation</td>
<td>Directly Leads to</td>
<td>Stellate cells, Activation</td>
</tr>
<tr>
<td>Stellate cells, Activation</td>
<td>Directly Leads to</td>
<td>Collagen, Accumulation</td>
</tr>
<tr>
<td>Collagen, Accumulation</td>
<td>Directly Leads to</td>
<td>Liver fibrosis, N/A</td>
</tr>
</tbody>
</table>

Protein, Alkylation Directly Leads to Cell death, N/A

How Does This Key Event Relationship Work

Alkylating agents are highly reactive chemicals that introduce alkyl radicals into biologically active molecules and thereby prevent their proper functioning. Many are used as antineoplastic agents, but most are very toxic, with carcinogenic, mutagenic, teratogenic, and immunosuppressant actions. Covalent protein alkylation by reactive electrophiles was identified as a key triggering event in chemical toxicity. Protein alkylation disturbs the cellular redox balance through interaction with glutathione, which leads to disruption of multiple biochemical pathways in exposed cells and is associated with
mitochondrial dysfunction which in turn can trigger the death of exposed cells via either apoptosis and/or necrosis. Hepatocytes are damaged via both covalent binding to liver proteins and lipid peroxidation accompanied by oxidative stress and collapse of mitochondrial membrane potential which triggers apoptotic cell death. Protein alkylation therefore also contributes to the development of oxidative stress, which plays a crucial role in fibrogenesis.  

Weight of Evidence

Biological Plausibility

cell injury caused by covalent binding is biologically plausible. The mechanistic relationship between MIE and KE consistent with established biological knowledge.  

Empirical Support for Linkage

There is exposure-dependent change in both events following exposure with temporal concordance.  

Uncertainties or Inconsistencies

Although covalent protein alkylation is a feature of many hepatotoxic drugs the overall extent of binding does not adequately distinguish toxic from non-toxic binding. Data from Codreanu et al. suggest that non-toxic covalent binding may largely be survivable damage to cytoskeletal components and other highly reactive protein targets, whereas toxic covalent binding produces lethal injury by targeting protein synthesis and catabolism and possibly mitochondrial electron transport. Future studies with appropriate probe molecules for toxic and non-toxic drugs could test these hypotheses and provide a better mechanistic basis for interpreting protein alkylation in drug safety evaluation.  

It is not known whether protein alkylation to certain proteins is required and whether particular proteins and various binding sites influence the further downstream process. Further we do not know whether there is a threshold and if this threshold would refer to the number of alkylation of a single protein or of a threshold number of proteins.  

Quantitative Understanding of the Linkage

Quantitative data are not available

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

References


Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20902

**Cell death, N/ A Directly Leads to Hepatic macrophages (Kupffer Cells), Activation**

**How Does This Key Event Relationship Work**

Damaged hepatocytes release reactive oxygen species (ROS), cytokines such as TGF-β1 and TNF-α, and chemokines which lead to oxidative stress, inflammatory signalling and finally activation of KCs. ROS generation in hepatocytes results from oxidative metabolism by NADH oxidase (NOX) and cytochrome 2E1 activation as well as through lipid peroxidation. Damaged liver cells trigger a sterile inflammatory response with activation of innate immune cells through release of damage-associated molecular patterns (DAMPs), which activate KCs through toll-like receptors and recruit activated neutrophils and monocytes into the liver. Central to this inflammatory response is the promotion ROS formation by these phagocytes. Upon initiation of apoptosis hepatocytes undergo genomic DNA fragmentation and formation of apoptotic bodies; these apoptotic bodies are consecutively engulfed by KCs and cause their activation. This increased phagocytic activity strongly up-regulates NOX expression in KCs, a superoxide producing enzyme of phagocytes with profibrogenic activity, as well as nitric oxide synthase (iNOS) mRNA transcriptional levels with consequent harmful reaction between ROS and nitric oxide (NO), like the generation of cytotoxic peroxinitrite (N2O3). ROS and/or diffusible aldehydes also derive from liver sinusoidal endothelial cells (LSECs) which are additional initial triggers of KC activation.

**Weight of Evidence**

**Biological Plausibility**

There is a functional relationship between cell injury/death and KC activation, consistent with established biological knowledge.


**Empirical Support for Linkage**

There is convincing theoretical evidence that hepatocyte injury and apoptosis causes KC activation, as well as inflammation and oxidative stress. But there are only limited experimental studies which could show that there is a direct relationship between these two events with temporal concordance. Specific markers for activated KCs have not been identified yet. KC activation cannot be detected by staining techniques since cell morphology does not change, but cytokines release can be measured (with the caveat that KCs activate spontaneously in vitro). Tukov et al. examined the effects of Kupffer cells cultured in contact with rat hepatocytes. They found that by adding Kupffer cells to the cultures they could mimic in vivo drug-induced inflammatory responses. Experiments on cells of the macrophage lineage showed significant aldehyde-induced stimulation of the activity of protein kinase C, an enzyme involved in several signal transduction pathways. Further, 4-Hydroxynonenal (HNE) was demonstrated to up-regulate TGF-β1 expression and synthesis in isolated rat KCs. Canbay et al could prove that engulfment of hepatocyte apoptotic bodies stimulated Kupffer cell generation of cytokines. [12] [13] [14] [15]

**Uncertainties or Inconsistencies**

The detailed mechanisms of the KC - hepatocyte interaction and its consequences for both normal and toxicant-driven liver responses remain to be determined. KC activation followed by cytokine release is associated in some cases with evident liver damage, whereas in others this event is unrelated to liver damage or may be even protective; apparently this impact is dependent on the quantity of KC activation; excessive or prolonged release of KC mediators can switch an initially protective mechanism to a damaging inflammatory response. Evidence suggests that low levels of cytokine release from KCs constitute a survival signal that protects hepatocytes from cell death and in some cases, stimulates proliferation. [3]

**Quantitative Understanding of the Linkage**

no quantitative data
Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

References

3. ↑ 30 31 Roberts et al, 2007, Role of the Kupffer Cell in Mediating Hepatic Toxicity and Carcinogenesis, Toxicological Sciences 96(1), 2-15

Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20981

Cell death, N/A Indirectly Leads to Stellate cells, Activation

How Does This Key Event Relationship Work

In addition to KC activation, damaged hepatocytes can also lead to activation of HSCs though the release of ROS, cytokines and chemokines. Engulfment of apoptotic bodies from hepatocytes can result in HSC activation and induce NOX expression in HSCs. DNA from apoptotic hepatocytes induces toll-like receptor 9 (TLR9)-dependent changes of HSCs that are consistent with late stages of HSC differentiation (activation), with up-regulation of collagen production and inhibition of platelet derived growth factor (PDGF)-mediated chemotaxis to retain HSCs at sites of cellular apoptosis. The release of latent TGF-beta complex into the microenvironment by damaged hepatocytes is likely to be one of the first signals for adjacent HSCs leading to their activation. As HSCs activation by hepatocytes, which is only a contributing factor and not the main route, is partly mediated by TGF-β1 this relationship is classified as indirect. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10]

Damaged hepatocytes also influence LSECs, which make an integral part of the hepatic reticulo-endothelial system and have a role in HSC activation. LSECs are morphologically identified by their fenestrations, which are transcytoplasmic canals arranged in sieve plates. In healthy liver, hepatocytes and HSCs maintain this phenotype of LSECs through release of vascular endothelial growth factor (VEGF). Differentiated (i.e. fenestrated) LSECs prevent HSC activation and promote reversal of activated HSC to quiescence, but LSEC lose this effect when they are de-differentiated due to liver injury. Preclinical studies have demonstrated that LSECs undergo defenestration as an early event that not only precedes liver fibrosis, but may also be permissive for it. Changes in LSEC differentiation might be an integral part of the development of fibrosis. Furthermore, in fibrosis LSECs become highly pro-inflammatory and secrete an array of cytokines and chemokines [11] [12] [13] [14] [15]

Weight of Evidence
**Biological Plausibility**


**Empirical Support for Linkage**

There is temporal concordance as stellate cell activation follows hepatic injury and there is experimental evidence for this KER. Canbay et al. could show that Fas-mediated hepatocyte injury is mechanistically linked to liver fibrogenesis. Markers of HSC activation were significantly reduced when apoptosis was prevented in Fas-deficient bile duct ligated mice. These findings (reduction of inflammation, markers of HSC activation, and collagen I expression) could be repeated by pharmacological inhibition of liver cell apoptosis using a pan-caspase inhibitor. Coulouarn et al found in a co-culture model that hepatocyte - stellate cell crosstalk engenders a permissive inflammatory microenvironment. [16] [17] [18]

**Uncertainties or Inconsistencies**

There are no inconsistencies

**Quantitative Understanding of the Linkage**

There are no quantitative data

**Evidence Supporting Taxonomic Applicability**

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

**References**

ligated mouse. J Pharmacol Exp Ther. 308, 1191-1196

Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20918

Hepatic macrophages (Kupffer Cells), Activation Directly Leads to TGFbeta1 expression, Up Regulation

How Does This Key Event Relationship Work

Following activation KCs become the main source for TGF-β1, the most potent profibrogenic cytokine, as well as a major source for inflammatory mediators and for ROS.

Expressed TNF-α (Tumor Necrosis Factor -alpha), TRAIL (TNF-related apoptosis-inducing ligand), and FasL (Fas Ligand) are pro-inflammatory active and also capable of inducing death receptor-mediated apoptosis in hepatocytes.

Activated KCs are an important source of ROS like superoxide (generated by NADPH oxidase (NOX). KCs express TNF-α, IL-1 (Interleukin-1) and MCP-1 (monocyte-chemoattractant protein-1), all being mitogens and chemoattractants for HSCs and induce the expression of platelet-derived growth factor (PDGF) receptors on hepatic stellate cells (HSCs) which further enhances HSCs proliferation

Weight of Evidence

Biological Plausibility

The functional relationship between these KEs is consistent with biological knowledge. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10]

Empirical Support for Linkage

Cytokine release is one of the features that define KC activation and there is sound empirical evidence for this KER. Experimental studies have shown enhanced cytokine gene expression by Kupffer cells in evolution of experimental liver injury. Northern blot analysis of freshly isolated Kupffer cells showed enhanced mRNA expression of three acute phase cytokines by the hepatic resident macrophages, TNF-α, IL-6 and TGF-β. [11] Experiments by Matsuoka and Tsukamoto already 1990 showed that KCs isolated from rat liver with alcoholic fibrosis express and release TGF-β1 and that this cytokine is largely responsible for the KC-conditioned medium-induced stimulation of collagen formation by HSCs. [11] [12]

Uncertainties or Inconsistencies

there are no inconsistencies

Quantitative Understanding of the Linkage
	no quantitative data

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

References

strategies. J Gastroenterol. 43, 419-428


Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20927

**TGFbeta1 expression, Up Regulation Directly Leads to Stellate cells, Activation**

**How Does This Key Event Relationship Work**

TGF-β1 is the most potent fibrogenic factor for HSCs. In response to TGF-β1, HSCs activate into myofibroblast-like cells, producing type I, III and IV collagen, proteoglycans like biglycan and decorin, glycoproteins like laminin, fibronec tin, tenasin and glycosaminoglycan. In the further course of events activated HSCs themselves express TGF-β1. TGF-β1 induces its own mRNA to sustain high levels in local sites of liver injury. The effects of TGF-β1 are mediated by intracellular signaling via Smad proteins. Smads 2 and 3 are stimulatory whereas Smad 7 is inhibitory. Smad1/5/8, MAP kinase and PI3 kinase are further signaling pathways in different cell types for TGF-β1 effects.

Concomitant with increased TGF-β production, HSC increase production of collagen. Connective tissue growth factor (CTGF) is a profibrogenic peptide induced by TGF-β, that stimulates the synthesis of collagen type I and fibronec tin and may mediate some of the downstream effects of TGF-β. It is upregulated during activation of HSC, suggesting that its expression is another determinant of a fibrogenic response to TGF-β. During fibrogenesis, tissue and blood levels of active TGF-β are elevated and overexpression of TGF-β1 in transgenic mice can induce fibrosis. Additionally, experimental fibrosis can be inhibited by anti-TGF-β treatments with neutralizing antibodies or soluble TbRs (TGF-β receptors).

**Weight of Evidence**

**Biological Plausibility**

There is good understanding and broad acceptance of this KER. There is good understanding and broad acceptance of this KER.

**Empirical Support for Linkage**

It is difficult to get experimental evidence in vitro for TGF-β1-induced HSC activation because HSCs undergo spontaneous activation when cultured on plastic; nevertheless qualitative empirical evidence for temporal and incidence concordance for this KER exists. Czaja et al could prove that treatment of cultured hepatic cells with TGF-β1 increased type I pro-collagen mRNA levels 13-fold due to post-transcriptional gene regulation. Tan et al. discovered that short TGF-β1 pulses can exert long-lasting effects on fibroblasts. Hepatic stellate cells activated in culture do not fully reproduce the changes in gene expression observed in vivo. De Minicis et al investigated gene expression changes in 3 different models of HSC activation and compared gene expression profiles in culture (mice HSCs in co-culture with KCs) and in vivo and did not find a proper correlation.

**Uncertainties or Inconsistencies**
No uncertainties that TGF-b1 activates HSCs.

**Quantitative Understanding of the Linkage**

no quantitative data

**Evidence Supporting Taxonomic Applicability**

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

**References**

1. ↑ Kisseleva and Brenner, 2007, Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis, Journal of Gastroenterology and Hepatology 22, Suppl. 1; S73-S78
4. ↑ Qi et al., 1999 Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat, Proc Natl Acad Sci U S A 96, 2345-2349
17. ↑ Liu X. et al.,(2006), Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. Liver Int. 26, 8-22
19. ↑ Tan A.B. et al. (2013). Cellular re- and de-programming by microenvironmental memory: why short TGF-β1 pulses can have long effects. Fibrogenesis Tissue Repair. 6, 12

Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20934

**Stellate cells, Activation Directly Leads to Collagen, Accumulation**

**How Does This Key Event Relationship Work**

Up-regulation of collagen (mainly typ I) synthesis following HSC activation is among the most striking molecular responses of HSCs to injury and is mediated by both transcriptional and post-transcriptional mechanisms. The half-life of collagen...
α1(I) mRNA increases 20-fold in activated HSCs compared with quiescent HSCs. Together with decreased matrix degradation (expression of degrading MMPs is down-regulated while their inhibitors TIMPs are up-regulated) ECM composition changes and further stimulates HSC activation and production of TGF-β1. Also increased mechanical stiffness of the ECM activates HSCs through integrin signalling. Monocytes and macrophages are involved in inflammatory actions by producing large amounts of NO and inflammatory cytokines such as TNF-α which have a direct stimulatory effect on HSC collagen synthesis. Chronic inflammation, hypoxia and oxidative stress re-activate EMT developmental programmes that converge in the activation of NF-kB. In response to growth factors (including PDGF, VEGF, thrombin) HSCs proliferate. Synthesis of TGF-α and TGF-β promotes activation of neighbouring quiescent hepatic stellate cells, whereas the release of HGF (hepatocyte growth factor) stimulates regeneration of adjacent hepatocytes. Monocytes and macrophages are involved in inflammatory actions by producing large amounts of nitric oxide and inflammatory cytokines such as TNFα which have a direct stimulatory effect on stellate cell collagen synthesis. Stellate cells are a key source of MMP-2 as well as increases in the specific MMP inhibitor molecules, TIMP-1 and TIMP-2, leading to a net decrease in protease activity, and therefore, more unopposed matrix accumulation. Moreover, an emerging role for TIMPs in regulating apoptosis suggests that their influence on liver homeostasis extends beyond that of direct effects on ECM turnover.

**Weight of Evidence**

**Biological Plausibility**

There is general acceptance that HSCs are collagen producing cells and key actors in fibrogenesis. The functional relationship between these KEs is consistent with biological knowledge.

**Empirical Support for Linkage**

It is difficult to stimulate sufficient collagen production and its subsequent incorporation into a pericellular matrix in vitro; therefore analytical methods have focused on measurement of pro-collagen secreted into culture medium or measurement of α-smooth muscle actin (α-SMA) expression, a marker of fibroblast activation. In primary culture, HSCs from normal liver begin to express α-SMA coincident with culture-induced activation.

**Uncertainties or Inconsistencies**

no inconsistencies

**Quantitative Understanding of the Linkage**

no quantitative data

**Evidence Supporting Taxonomic Applicability**

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

**References**

2. ↑ 2.0 2.1 Milani et al., 1994, Differential expression of matrix-metalloproteinase-1 and -2 genes in normal and fibrotic human liver. Am J Pathol.;144:528-537
4. ↑ 4.0 4.1 Kolios G. et al., (2006), Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol. 12, 7413-7420
Collagen, Accumulation Directly Leads to Liver fibrosis, N/ A

How Does This Key Event Relationship Work

Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen. Liver fibrosis results from an imbalance between the deposition and degradation of extracellular matrix (ECM) and a change of ECM composition; the latter initiates several positive feedback pathways that further amplify fibrosis. With chronic injury, there is progressive substitution of the liver parenchyma by scar tissue. Deposition of collagen in the liver progressively disrupts the normal hepatic architecture so that the normal relationship between vascular inflow and outflow is destroyed and the normal collagen content around hepatic sinusoids in regenerating nodules becomes modified. Advanced liver fibrosis results in cirrhosis. [1] [2]

Weight of Evidence

Biological Plausibility

By definition liver fibrosis is the excessive accumulation of extracellular matrix proteins that are produced by HSCs. The KER between this KE and the AO is undisputed. [1] [2]

Empirical Support for Linkage

There is a smooth transition from ECM accumulation to liver fibrosis without a definite threshold and plenty in vivo evidence exists that ECM accumulation is a pre-stage of liver fibrosis [1] [2]

Uncertainties or Inconsistencies

no inconsistencies

Quantitative Understanding of the Linkage

no quantitative data

Evidence Supporting Taxonomic Applicability

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>Strong</td>
<td>NCBI</td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>Strong</td>
<td>NCBI</td>
</tr>
</tbody>
</table>

References


Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20944

Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=20946

Overall Assessment of the AOP

Assessment of the Weight-of-Evidence supporting the AOP

Concordance of dose-response relationships

This is a qualitative description of the pathway; the currently available literature does not provide quantitative information on dose-response relationships. But there is empirical evidence to support that a change in KEup leads to an appropriate change in the respective KEdown.

Temporal concordance among the key events and adverse outcome

Qualitative empirical evidence for temporal concordance between the individual KEs sequences leading to the adverse outcome exists.

Strength, consistency, and specificity of association of adverse outcome and initiating event

The scientific evidence on the linkage between initiating event and adverse outcome has been described. The ample literature is consistent in describing this association between AO and MIE

Biological plausibility, coherence, and consistency of the experimental evidence

The available data supporting the AOP are logic, coherent and consistent with established biological knowledge.

Alternative mechanism(s) that logically present themselves and the extent to which they may distract from the postulated AOP

It should be noted that alternative mechanism(s) of action, if supported, require a separate AOP.

There are some other important fibrogenic signalling pathways that influence HSC activation and fibrogenesis without constituting another AOP:

Adipokine pathways

Adipokines are secreted mainly by adipose tissue, but also by resident and infiltrating macrophages and are increasingly recognised as mediators of fibrogenesis.

Leptin promotes HSC fibrogenesis and enhances TIMP-1 expression and further acts as a pro-fibrotic through suppression of peroxisome proliferator-activated receptor- \( \bullet \) (PPAR\( \bullet \)), an anti-fibrogenic nuclear receptor that can reverse HSC activation. The expression of leptin receptor is up-regulated during HSC activation and leptin activity is therefore increased through enhanced signaling. Downstream effects include increased release of TGF-b1 from KCs. The counter-regulatory hormone adiponectin is reduced in hepatic fibrosis. \[1\] \[2\]

Neuroendocrine pathways

The fibrogenic function of HSCs is also influenced by neurochemical and neurotrophic factors. Upon chronic liver injury, the local neuroendocrine system is up-regulated, and activated HSCs express specific receptors, most prominently those regulating cannabinoid signaling. Activated HSCs are additionally a key source of the endogenous cannabinoid, 2-Arachidonylglycerol (2-AG), which drives increased (cannabinoid-receptor) CB 1 signalling. Stimulation of the CB1 receptor is profibrogenic, whereas the CB2 receptor is anti-fibrotic and hepatoprotective. Opioid signaling increases proliferation and collagen production in HSCs. Serotonin has a pro-fibrotic effect that synergizes with PDGF signaling. Also thyroid hormones
enhance activation of HSC (through increased p75 neurotrophin receptor (p75NTR) and activation of Rho), thereby accelerating the development of liver fibrosis. [3] [2] [1]

Renin–angiotensin pathway

Angiotensin II (Ang II) is a pro-oxidant and fibrogenic cytokine that stimulates DNA synthesis, cell migration, procollagen α1(I) mRNA expression, and secretion of TGF-β1 and inflammatory cytokines. These fibrogenic actions are mediated by NOX. [4] [2] [1] [5]

Uncertainties, inconsistencies and data gaps

The description is plausible, but purely qualitative; the addition of quantitative data on dose response-relationships and temporal sequences is needed and would substantially improve its applicability.

Protein alkylation is a broad, non-specific MIE. Covalent protein alkylation is a feature of many hepatotoxic drugs but the overall extent of binding does not adequately distinguish toxic from non-toxic binding. For this AOP it is unclear whether protein alkylation per se is sufficient to start the pathway or whether alkylation to specific proteins or families of proteins needs to be affected and whether various binding sites influence the further downstream process. The identification and specification of the targeted biomolecules is needed for the structural definition of chemical initiators and consecutively for profiling and categorising of chemicals related to the initiation of this AOP. Likewise it is necessary for the establishment of a distinct relationship with the next downstream event. Further it is unknown whether there is a threshold and if this threshold would refer to the number of alkylation of a single protein or of a threshold number of proteins. Future studies could provide a better mechanistic basis for interpreting protein alkylation in chemical safety evaluation.

By definition an AOP has only one MIE and one final AO, the two anchor points of the AOP that have to be clearly defined. Any other MIE that leads to cell injury and further to liver fibrosis via the same downstream KEs would constitute another AOP. There are various types of liver injury that are caused by different agents, initiated by various MIEs and finally lead to fibrosis via the same described pathway; therefore the question arises whether hepatocyte injury itself, independently from the cause of injury, might be the initiating event for this pathway to fibrosis. Obviously hepatocyte injury does not inevitably lead to fibrosis in all cases and there is a wide range of hepatotoxic chemicals (like Acetaminophen, Aflatoxin or Chlorpromazine) for which liver fibrosis cannot be observed. Apoptosis, necrosis, transdifferentiation/transformation and repair/regeneration, all these might occur in response to cellular stressors and the difference in progression to liver fibrosis might lie in these various cellular responses. There is increasing evidence for apoptosis being the main fibrogenic trigger. Yet, both necrosis and apoptosis are often present simultaneously and necrosis may only represent the more severe cellular response to stronger damaging stimuli. It also might well be that hepatocyte insult/injury, rather than death is sufficient to trigger fibrosis and the key question would then be whether there are fibrosis-specific features of cell injury. It could be rather the amount (quantitative difference) than the kind (qualitative difference) of cell injury that matters. The rate of cell injury/death, i.e. the amount of injury within a certain time frame could be another plausible initiating parameter, as fibrosis is resulting from chronic injury. Assuming hepatocyte injury being the crucial key event without which fibrosis could not occur via this AOP, then simple investigation of in vitro hepatotoxicity could provide relevant information for potential fibrosis prediction without the need of highly elaborated cell models.

Assessment of the quantitative understanding of the AOP

See above

Weight of Evidence Summary

<table>
<thead>
<tr>
<th>Event</th>
<th>Description</th>
<th>Triggers</th>
<th>Weight of Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, Alkylation</td>
<td>Directly Leads to</td>
<td>Cell death, N/A</td>
<td>Strong</td>
</tr>
<tr>
<td>Cell death, N/A</td>
<td>Directly Leads to</td>
<td>Hepatic macrophages (Kupffer Cells), Activation</td>
<td>Strong</td>
</tr>
<tr>
<td>Cell death, N/A</td>
<td>Indirectly Leads to</td>
<td>Stellate cells, Activation</td>
<td>Strong</td>
</tr>
<tr>
<td>Hepatic macrophages (Kupffer Cells), Activation</td>
<td>Directly Leads to</td>
<td>TGFbeta1 expression, Up Regulation</td>
<td>Strong</td>
</tr>
<tr>
<td>TGFbeta1 expression, Up Regulation</td>
<td>Directly Leads to</td>
<td>Stellate cells, Activation</td>
<td>Strong</td>
</tr>
</tbody>
</table>
Alkylating agents are highly reactive chemicals that introduce alkyl radicals into biologically active molecules and thereby prevent their proper functioning.

Essentiality of the MIE is high.

Covalent protein alkylation by reactive electrophiles was identified as a key triggering event in chemical toxicity over 40 years ago. These reactions remain a major cause of chemical-induced toxicity. [6] [7]

Covalent binding to liver proteins and oxidative stress can directly affect cell or influence signalling pathways, finally leading to necrotic or apoptotic cell death.

Essentiality of KE 1 is high.

Up-regulated apoptosis of hepatocytes is increasingly viewed as a nexus between liver injury and fibrosis. Pharmacological inhibition of liver cell apoptosis attenuates liver injury and fibrosis suggesting a critical role for hepatocyte apoptosis in the initiation of HSC activation and hepatic fibrogenesis. [8] [9] [10] [11] [12] [13] [14] [15] [16]

Activated KCs are a major source of inflammatory mediators including cytokines, chemokines, lysosomal and proteolytic enzymes and a main source of TGF-β, as well as a major source of ROS.

Essentiality of KE 2 is high.

Probably there is a threshold of KC activation and release above which liver damage is induced. Pre-treatment with gadolinium chloride (GdCl), which inhibits KC function, reduced both hepatocyte and sinusoidal epithelial cell injury, as well as decreased the numbers of macrophages appearing in hepatic lesions and inhibited TGF-1 mRNA expression in macrophages. Experimental inhibition of KC function or depletion of KCs appeared to protect against liver injury from the alkylating agent melphalan , the chemical thioacetamide and the immunostimulants concanavalin A and Pseudomonas exotoxin. [17] [4] [18] [19] [16] [20] [21] [22]
<table>
<thead>
<tr>
<th>KE 3</th>
<th>TGF-β1 expression</th>
<th>TGF-β1 is the most potent profibrogenic cytokine and plays a central role in fibrogenesis, mediating a cross-talk between parenchymal, inflammatory and collagen expressing cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KE 4</td>
<td>hepatic stellate cell (HSC) activation</td>
<td>HSC activation (in response to TGF-β1) means a transdifferentiation from a quiescent vitamin A–storing cell to a proliferative and contractile myofibroblast and is the dominant event in liver fibrosis. Activated HSCs (myofibroblasts) are the primary collagen producing cell, the key cellular mediators of fibrosis and a nexus for converging inflammatory pathways leading to fibrosis.</td>
</tr>
<tr>
<td>KE 5</td>
<td>collagen accumulation</td>
<td>Excess ECM (extracellular matrix) deposition and changes in ECM composition.</td>
</tr>
<tr>
<td>Adverse Outcome</td>
<td>liver fibrosis</td>
<td>Excessive deposition of ECM proteins occurs as a result of repeated cycles of hepatocytes injury and repair and results in liver fibrosis.</td>
</tr>
</tbody>
</table>

Essentiality of KE 3 is high.  
TGF-β1 is considered the most potent pro-fibrogenic cytokine and several reviews assign this cytokine a central role in fibrogenesis, especially in HSC activation. Strategies aimed at disrupting TGF-β1 expression or signaling pathways are extensively being investigated because blocking this cytokine may not only inhibit matrix production, but also accelerate its degradation. Animal experiments using different strategies to block TGF-β1 have demonstrated significant anti-fibrotic effect for liver fibrosis. Experimental fibrosis can be inhibited by anti-TGF-β treatments with neutralizing antibodies or soluble TbRs (TGF-β receptors). [23] [24] [25] [26] [27]

Essentiality of KE 4 is high.  
Experimental inhibition of HSC activation prevents fibrosis. [4] [28] [29] [30] [31] [32]

Essentiality of KE 5 is high.  
Continuing imbalance between the deposition and degradation of ECM is a pre-requisite of liver fibrosis; therefore this KE is essential for the AO. [1]

It is generally accepted that any chronic form of liver damage, including any chemical causing sub-massive hepatocellular injury, can result in myofibroblast activation, leading to hepatic fibrosis and cirrhosis in humans [33] [34] [35] [36] [37].
Nevertheless a short overview is given below.

| Associated Event | Hepatic fibrosis is commonly preceded by chronic inflammation and persistent inflammation has been associated with progressive hepatic fibrosis. Hepatic inflammation is a driver of hepatic fibrosis as the whole fibrinogenic cascade is initiated and maintained by inflammatory mediators and inflammatory and fibrogenic cells stimulate each other in amplifying fibrosis. Damaged hepatocytes release inflammatory cytokines that activate KCs and stimulate the recruitment of inflammatory cells which produce profibrotic cytokines and chemokines that further activate fibroblastic cells. Activated HSCs secrete various cytokines (like macrophage colony-stimulating factor (M-CSF), MCP-1 and IL-6) and inflammatory chemokines, they interact directly with immune cells through expression of adhesion molecules (mediated by TNF-α and facilitating the recruitment of inflammatory cells), and they modulate the immune system through antigen presentation. Signaling of HSCs in response to either LPS or endogenous TLR4 ligands down-regulates the protein activin membrane-bound inhibitor (BAMBI), a transmembrane suppressor of TGF-β1. Other inflammatory cells regulating progression and resolution of fibrosis include T-cells, dendritic cells, LSECs and natural killer cells (NK), which exert an anti-fibrotic activity by inducing HSC apoptosis through production of IFN γ. Chronic inflammatory response is often accompanied simultaneously by tissue destruction and repair. Activated inflammatory cells represent a major source of oxidative stress-related molecules. |
| Essentaility of inflammation is high. |
| Suppression of inflammatory activity by eliminating the etiological agent (e.g. a virus) or dampening the immune response (lymphocytic proliferation and infiltration) can halt and even reverse the fibrotic process. |

| Associated Event | Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation and plays a crucial role in liver fibrogenesis by inducing hepatocyte apoptosis and activation of KCs and HSCs. Oxidative stress-related molecules act as mediators to modulate tissue and cellular events responsible for the progression of liver fibrosis. ROS, including superoxide, hydrogen peroxide, hydroxyl radicals and aldehydic end products may be derived from hepatocytes (generated through cytochrome P450, lipid peroxidation), as well as from activated KCs, other inflammatory cells and HSCs (by NOX). Excessive levels of ROS can lead to hepatocellular injury and death. Under conditions of oxidative stress macrophages are activated which leads to a more enhanced inflammatory response. Oxidative stress can activate a variety of transcription factors like NF-κB, PPAR-γ which may further lead to increased gene expression for the production of growth factors, inflammatory cytokines and chemokines. |
| Essentaility of oxidative stress is moderate. |
| Oxidative stress-related molecules act as mediators to modulate tissue and cellular events responsible for the progression of liver fibrosis. Hence reactive oxidant species likely contribute to both onset and progression of fibrosis, being simultaneously cause and consequence of the observed condition. |

| Support for Biological Plausibility of KERs | Biological Plausibility of the MIE => KE1 is high. |
| Biological Plausibility of KE1 => KE2 is high |

| MIE => KE1 | Hepatocytes are damaged by alkylating agents via both covalent binding to liver proteins and lipid peroxidation accompanied by oxidative stress and collapse of mitochondrial membrane potential which triggers apoptotic cell death. |
| Biological Plausibility of the MIE => KE1 is high. There is a mechanistic relationship between MIE and KE 1 consistent with established biological knowledge. |

| KE1 => KE2 | Damaged hepatocytes release reactive oxygen species (ROS), cytokines and chemokines which lead to oxidative stress, inflammatory signaling and activation of KCs. Apoptotic hepatocytes undergo genomic DNA fragmentation and formation of apoptotic bodies. Upon engulfment of apoptotic bodies KCs are activated. Liver cells trigger a sterile inflammatory |
| Biological Plausibility of KE1 => KE2 is high |
| There is a functional relationship between KE 1 and KE 2 consistent with |
response with activation of innate immune cells through release of damage-associated molecular patterns (DAMPs). Through toll-like receptors KCs are additionally activated.

| KE 1 => KE 4 | Biological Plausibility of KE1 => KE4 is high |
| KE 2 => KE 3 | Biological Plausibility of KE2 => KE3 is high |
| KE 3 => KE 4 | Biological Plausibility of KE3 => KE4 is high |
| KE 4 => KE 5 | Biological Plausibility of KE4 => KE5 is high |
| KE 5 => AO | Biological Plausibility of KE5 => AO is high |

Like KCs also HSCs are activated by damaged hepatocytes through the release of ROS, cytokines and chemokines and upon engulfment of apoptotic bodies from apoptotic hepatocytes. DNA from apoptotic hepatocytes induces toll-like receptor 9 (TLR9)-dependent changes of HSCs that are consistent with late stages of HSC differentiation (activation), with up-regulation of collagen production and inhibition of platelet derived growth factor (PDGF)-mediated chemotaxis to retain HSCs at sites of cellular apoptosis. The release of latent TGF-β complex into the microenvironment by damaged hepatocytes is likely to be one of the first signals for adjacent HSCs leading to their activation.

Following activation KCs become a main source of TGF-β1, the most potent profibrogenic cytokine, as well as a major source of inflammatory mediators, chemokines, and ROS.

TGF-β1 activates HSCs, i.e. stimulates cell proliferation, matrix synthesis, and release of retinoids by HSCs and is the most potent fibrogenic factor for HSCs.

In response to TGF-β1 activated HSCs up-regulate collagen synthesis. Together with decreased matrix degradation ECM composition changes and further stimulates HSC activation and production of TGF-β1, which further promotes activation of neighbouring quiescent HSCs.

Excessive accumulation of ECM proteins leads to disruption of normal liver tissue.

HSCs activation by hepatocytes is only a contributing factor and not the main route; partly it is mediated by TGF-β1; therefore this relationship is classified as indirect. Nevertheless there is a functional relationship between KE 1 and KE 4 consistent with established biological knowledge.

The functional relationship between KE 2 and KE 3 is consistent with biological knowledge.

There is good understanding and broad acceptance of the KER between KE 3 and KE 4.

The functional relationship between KE 4 and KE 5 is consistent with biological knowledge and generally accepted.

By definition liver fibrosis is the excessive accumulation of ECM proteins leading to disruption of normal liver tissue.
**Empirical Support for KERs**

There is a need for more advanced in vitro models systems for chemical-induced hepatotoxicity to study intercellular signalling and dose-response data on KERs. Nevertheless some empirical evidence exists to support that a change in KEup leads to an appropriate change in the respective KEdown.

| MIE => KE 1 | It is general accepted knowledge that alkylating chemicals damage cells. Although covalent protein alkylation is a feature of many hepatotoxic drugs the overall extent of binding does not adequately distinguish toxic from non-toxic binding. It is not known whether protein alkylation to certain proteins is required and whether particular proteins and various binding sites influence the further downstream process. Further we do not know whether there is a threshold and if this threshold would refer to the number of alkylation of a single protein or of a threshold number of proteins. | Empirical Support of the MIE => KE 1 is moderate. There is exposure-dependent change in both events following exposure with temporal concordance. **[73]** **[74]** **[75]** **[76]** **[6]** |
| KE 1 => KE 2 | Specific markers for activated KCs have not been identified yet. KC activation cannot be detected by staining techniques since cell morphology does not change, but cytokines release can be measured (with the caveat that KCs activate spontaneously in vitro). Tukov et al. examined the effects of KCs cultured in contact with rat hepatocytes. They found that by adding KCs to the cultures they could mimic in vivo drug-induced inflammatory responses. Canbay et al. could prove that engulfment of hepatocyte apoptotic bodies stimulated cytokine expression by KCs. | Empirical Support of the KE 1 => KE 2 is moderate. There are limited experimental studies which could show that there is a direct relationship between these two events with temporal concordance. **[77]** **[78]** **[79]** **[13]** |
| KE 1 => KE 4 | Canbay et al. could show that Fas-mediated hepatocyte injury is mechanistically linked to liver fibrogenesis. Markers of HSC activation were significantly reduced when apoptosis was prevented in Fas-deficient bile duct ligated mice. These findings (reduction of inflammation, markers of HSC activation, and collagen I expression) could be repeated by pharmacological inhibition of liver cell apoptosis using a pan-caspase inhibitor. Watanabe et al. could demonstrat in vitro that DNA from apoptotic hepatocytes acts as an important mediator of HSC differentiation by providing a stop signal to mobile HSCs when they have reached an area of apoptosing hepatocytes and inducing a stationary phenotype-associated up-regulation of collagen production. Coulouarn et al found in a co-culture model that hepatocyte - HSC crosstalk engenders a permissive inflammatory microenvironment. | Empirical Support of the KE 1 => KE 4 is moderate. There is experimental evidence for this KER. **[12]** **[16]** **[80]** **[81]** |
| KE 2 => KE 3 | Experiments by Matsuoka and Tsukamoto already 1990 showed that KCs isolated from rat liver with alcoholic fibrosis express and release TGF-β1 and that this cytokine is largely responsible for the KC-conditioned medium-induced stimulation of collagen formation by HSCs. Accumulated CD11b1 macrophages are critical for activating HSCs (via expression of TGF-β1) (Chu et al, 2013) | Empirical Support of the KE 2 => KE 3 is moderate. Cytokine release is one of the features that define KC activation and there is sound empirical evidence for this KER. **[68]** **[82]** |
Czaja et al could prove that treatment of cultured hepatic cells with TGF-β1 increased type I pro-collagen mRNA levels 13-fold due to post-transcriptional gene regulation. Tan et al. discovered that short TGF-β1 pulses can exert long-lasting effects on fibroblasts. Difficulties are that HSCs cultured on plastic undergo spontaneous activation and HSCs activated in culture do not fully reproduce the changes in gene expression observed in vivo. De Minicis et al investigated gene expression changes in 3 different models of HSC activation and compared gene expression profiles in culture (mice HSCs in co-culture with KCs) and in vivo and did not find a proper correlation.

Empirical Support of the KE 3 => KE 4 is moderate.
Qualitative empirical evidence with temporal and incidence concordance for this KER exists. [83] [84] [85] [86]

It is difficult to stimulate sufficient production of collagen and its subsequent incorporation into a pericellular matrix in vitro; therefore analytical methods have focused on measurement of pro-collagen secreted into culture medium or measurement of α-smooth muscle actin (α-SMA) expression, a marker of fibroblast activation. In primary culture, HSCs from normal liver began to express smooth muscle alpha actin coincident with culture-induced activation.

Empirical Support of the KE 4 => KE 5 is moderate.
It is general accepted knowledge that activated HSCs (=myofibroblasts) are collagen-producing cells. [87] [88]

Liver fibrosis results from chronic damage in conjunction with the accumulation of ECM proteins, which distorts the hepatic architecture by forming a fibrous scar. The onset of liver fibrosis is usually insidious and progression to cirrhosis occurs after an interval of 15–20 years.

Empirical Support of the KE 5 => AO is high.
There is a smooth transition from ECM accumulation to liver fibrosis without a definite threshold and plenty in vivo evidence exists that ECM accumulation is a pre-stage of liver fibrosis [44].

**Essentiality of the Key Events**

<table>
<thead>
<tr>
<th>Molecular Initiating Event</th>
<th>Support for Essentiality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, Alkylation</td>
<td>Strong</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Event</th>
<th>Support for Essentiality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death, N/A</td>
<td>Strong</td>
</tr>
<tr>
<td>Hepatic macrophages (Kupffer Cells), Activation</td>
<td>Strong</td>
</tr>
<tr>
<td>TGFβ1 expression, Up Regulation</td>
<td>Strong</td>
</tr>
<tr>
<td>Stellate cells, Activation</td>
<td>Strong</td>
</tr>
<tr>
<td>Collagen, Accumulation</td>
<td>Strong</td>
</tr>
<tr>
<td>Oxidative Stress, Increase</td>
<td>Moderate</td>
</tr>
<tr>
<td>Chronic Inflammation, Increase</td>
<td>Strong</td>
</tr>
</tbody>
</table>

The essentiality of each of the KEs for this AOP was rated high as there is much experimental evidence that the blocking of one KE prevents (or attenuates where complete blocking is not possible) the next downstream KE and therefore the whole...
AOP. Much evidence arises from preclinical research for antifibrotic agents, which is mainly based on the interference with or blockade of a key event. For details see the table above.

### Quantitative Considerations

<table>
<thead>
<tr>
<th>Event</th>
<th>Description</th>
<th>Triggers</th>
<th>Quantitative Understanding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, Alkylation</td>
<td>Directly Leads to</td>
<td>Cell death, N/A</td>
<td></td>
</tr>
<tr>
<td>Cell death, N/A</td>
<td>Directly Leads to</td>
<td>Hepatic macrophages (Kupffer Cells), Activation</td>
<td></td>
</tr>
<tr>
<td>Cell death, N/A</td>
<td>Indirectly Leads to</td>
<td>Stellate cells, Activation</td>
<td></td>
</tr>
<tr>
<td>Hepatic macrophages (Kupffer Cells), Activation</td>
<td>Directly Leads to</td>
<td>TGFbeta1 expression, Up Regulation</td>
<td></td>
</tr>
<tr>
<td>TGFbeta1 expression, Up Regulation</td>
<td>Directly Leads to</td>
<td>Stellate cells, Activation</td>
<td></td>
</tr>
<tr>
<td>Stellate cells, Activation</td>
<td>Directly Leads to</td>
<td>Collagen, Accumulation</td>
<td></td>
</tr>
<tr>
<td>Collagen, Accumulation</td>
<td>Directly Leads to</td>
<td>Liver fibrosis, N/A</td>
<td></td>
</tr>
</tbody>
</table>

More advanced in vitro models systems are needed to study chemical-induced hepatotoxicity. Modulations of hepatotoxicity by intercellular signalling cannot be addressed in primary cultures of hepatocytes alone but require co-cultures of different liver cell types. Various co-cultures systems with two or more different liver cell types are currently being developed, but quantitative data on KERs are not available yet.

### Applicability of the AOP

<table>
<thead>
<tr>
<th>Life Stage</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not Otherwise Specified</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific Name</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>Homo sapiens</td>
<td>NCBI</td>
<td></td>
</tr>
<tr>
<td>rodents</td>
<td></td>
<td>NCBI</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th>Evidence</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unspecific</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The described AOP is valid for both sexes and any lifestage. This pathway description is based on studies of formation and progression of fibrosis in human patients. Findings also suggest common conserved pathways across different species which initiate and promote liver fibrosis. Animal models are used to study fibrogenesis and CCl4 intoxication in rats and mice is probably the most widely studied and therefore best characterised model with respect to histological, biochemical, cell, and molecular changes associated with the development of fibrosis.

### Considerations for Potential Applications of the AOP (optional)

A sufficiently detailed description of the AOP to liver fibrosis might support chemical risk assessment by indicating early (upstream) markers for downstream events and facilitate a testing strategy without the need for an elaborated cell model.

### References


33
8. ↑ 8.0 8.1 8.2 Malhi, H. et al., (2010), Hepatocyte death: a clear and present danger. Physiol Rev. 90, 1165-1194
10. ↑ 10.0 10.1 10.2 Orrenius S. et al.,(2011), Cell Death Mechanisms and Their Implications in Toxicology. Toxicol. Sci. 119, 3-19
17. ↑ 17.0 17.1 17.2 17.3 17.4 17.5 Kolios G. et al., (2006), Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol. 12, 7413-7420
18. ↑ 18.0 18.1 Roberts R.A. et al. (2007), Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. Toxicol Sci. 96, 2-15
23. ↑ 23.0 23.1 Liu X. et al.,(2006), Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. Liver Int. 26, 8-22
25. ↑ Cheng K et al., (2009), TGF-β1 gene silencing for treating liver fibrosis. Mol Pharm. 6, 772-779
27. ↑ 27.0 27.1 Qi Z. et al., (2010), Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. Proc Natl Acad Sci. 96,2345-2349
31. ↑ Son G. et al., (2009). Inhibition of phosphatidylinositol 3-kinase signaling in hepatic stellate cells blocks the progression of hepatic fibrosis. Hepatology. 50, 1512-1523


45. Guo J. and Friedman S.L., (2010), Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis, Fibrogenesis & Tissue Repair 3, 21


60. Roth S. et al., (1998). (Latent) transforming growth factor beta in liver parenchymal cells, its injury-dependent release, and paracrine effects on rat hepatic stellate. Hepatology. 27, 1003-1012


82. Tan A.B. et al. (2013). Cellular re- and de-programming by microenvironmental memory: why short TGF-β1 pulses can have long effects. Fibrogenesis Tissue Repair. 6, 12

Retrieved from https://aopkb.org/aopwiki/index.php/?oldid=21154

This snapshot was created on March 03, 2015 at 19:06 EST