Mini-Review

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Using silkworms to establish alternative animal models for evaluation of drug-induced tissue injury

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Summary **Evaluation of tissue injury induced by chemicals is crucial to drug development. Mice and rats, which are effectively used to analyze drug-induced tissue injury, present problems in terms of cost and ethical issues. Although alternative methods have been developed using *in vitro* techniques or invertebrates, evaluation of ADME and the size of animals are still issues that need to be addressed. Use of silkworms can resolve these problems. Silkworms have pharmacokinetic characteristics similar to those of mammals. Injection of various hepatotoxic chemicals also leads to elevated alanine aminotransferase (ALT) activity in the hemolymph of silkworms. Furthermore, transparent transgenic silkworms expressing GFP have been produced to facilitate continuous analysis without the need to collect hemolymph. Analyses using this silkworm have indicated that the intensity of GFP fluorescence observed on the body surface of the silkworm decreases in a time- and dose-dependent manner when hepatotoxic chemicals are injected. These results suggest that the silkworms can serve as alternative animal model for evaluation of drug-induced tissue injury.**

Keywords: Drug-induced tissue injury, alternative animal model, silkworm, hepatotoxicity, fluorescence

1. Introduction

Tissue injury induced by chemicals in mammals, including humans, results in the rapid onset of severe dysfunction of the organs involved in detoxification, as exemplified by fulminant hepatic failure (*1*). Therefore, predicting tissue injury, and especially hepatotoxicity, is an important aspect of novel drug discovery.

When novel therapeutic medicines are developed, *in vivo* trials using animal models are essential to predicting toxicity and drug disposition in the human body. Mice and rats are used to evaluate the toxicity of synthesized compounds and natural medicines (*2,3*). However, the use of mammals as experimental models presents a number of problems, such as cost and ethical issues (*4*). *In vitro* assay systems using human hepatocytes have been developed in order to solve these problems (*5,6*). Toxicogenomic systems should be effective at predicting hepatotoxicity based on the expression of various genes that respond hepatotoxicity (*7,8*). However, these *in vitro* assay systems still present problems in terms of the collection of mammalian cells and differences in conditions from those *in vivo*. An alternative animal model is therefore needed to overcome these problems (Table 1).

Although the invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans* are used in experiments in general biology, injection of a precise volume of a sample to determine the LD_{50} in pharmacological analyses is difficult because invertebrates have small bodies (*9,10*). The silkworm has been proposed as an invertebrate model in which to quantitatively administer a drug solution *via* injection with a typical syringe (*11,12*). Another advantage of using silkworms as an animal model in pharmacological research relates to the characteristics of drug metabolism. Silkworms metabolize chemicals *via* processes similar to those found in mammals; in phase I, reactions are catalyzed by cytochrome P450s and metabolites are conjugated in phase II (*13*). The LD_{50} of various cytotoxic compounds in silkworms closely match similar values in mammals

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(*13*). Furthermore, antibiotics have similar therapeutic effects in silkworms and mammals (*11,14,15*). These results indicate that silkworms may be suitable for screening drug candidates that have therapeutic effects in mammals.

There are various methods of evaluating hepatotoxicity induced by drugs (*16,17*). Alanine aminotransferase (ALT) activity in the blood of mammals is frequently measured to evaluate liver function in basic research and routine practice (*18*). The level of ALT

Table 1. Advantages and disadvantages of in vitro and in vivo models for evaluation of drug-induced tissue injury

Model	Advantages/disadvantages	*Ref*
Hepatocytes (*in vitro*)	Advantages: high throughput analysis, cell imaging system, fewer ethical issues. Disadvantages: reproducibility of *in vivo* pharmacokinetics such as ADME, collection of hepatocytes from mammals.	(*5-8*)
Small invertebrates (*in vivo*)	Advantages: high throughput analysis, clarified genetic background, fewer ethical issues. Disadvantages: difficulty of quantitative sample injection.	(*9,10*)
Silkworms (*in vivo*)	Advantages: high throughput analysis, pharmacokinetic characteristics similar to those of mammals, fewer ethical issues. Disadvantages: less knowledge regarding metabolic machinery of extraneous chemicals.	(*20,25*)
Vertebrates (*in vivo*)	Advantages: pharmacokinetic characteristics similar to those of humans. Disadvantages: cost and ethical issues, need for a large amount of samples.	(*4*)

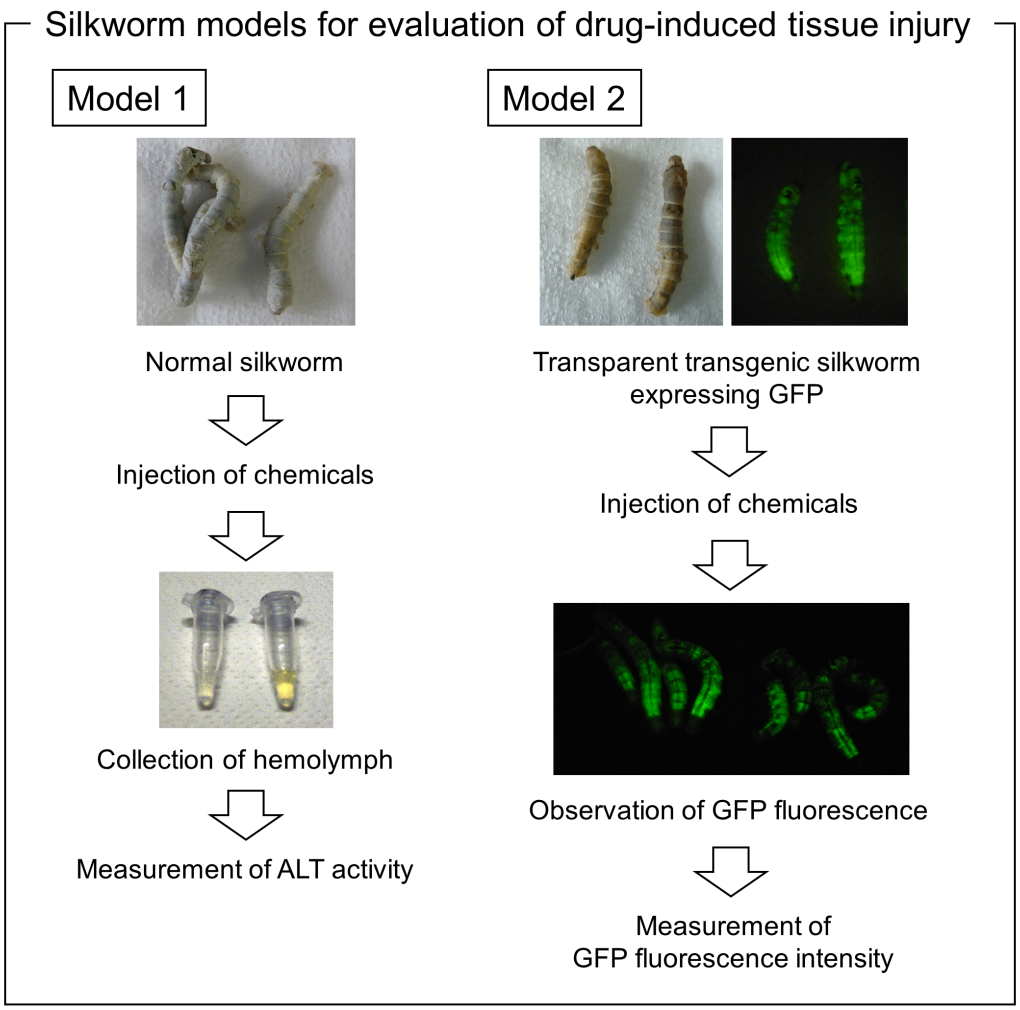

Figure 1. Protocols for silkworm models for evaluation of drug-induced tissue injury. Evaluation of tissue injury in model 1 is based on the level of ALT activity while evaluation in model 2 is based on the intensity of GFP fluorescence.

activity in human blood is considered to be a highly sensitive and fairly specific preclinical and clinical biomarker of cytotoxicity or hepatotoxicity, so many pharmaceutical studies have used the level of ALT in the blood of mammals to evaluate the hepatotoxic effects of natural products or newly synthesized chemicals (*18,19*). Therefore, an alternative animal model that allows evaluation of the level of ALT activity needs to be established. In a previous study by the current authors, ALT activity was measured in the hemolymph of silkworms using usual enzymatic methods (*20*). The ALT level in the hemolymph of silkworm larvae rose with injection of CCl_4, which is generally used as a model compound for examination of hepatotoxic effects in mammals (*20*). Elevated ALT activity in the hemolymph of silkworms has been induced with various hepatotoxic chemicals such as acetaminophen, tetracycline, ketoconazole, and D-galactosamine (*20*). These results suggest that the silkworm can be used to evaluate hepatotoxicity by measuring the level of ALT activity in hemolymph.

As described above, the silkworm model can be used to perform various analyses in pharmacological research, such as evaluations of toxicity and therapeutic action. However, those silkworm models required an invasive approach to obtain hemolymph or to collect tissue. Thus, establishment of a new silkworm model has focused on a model that allows external observation of a silkworm while collecting continuous data from the same animal. Recently, *in vivo* imaging techniques based on fluorescence have progressed markedly and various animal models have been developed (*21-23*). A transgenic silkworm that displays GFP fluorescence has been developed using the GAL4-UAS system (*24*). The fluorescence of the tissue expressing GFP in this transgenic silkworm is consistently visible for a prolonged period as a result of exposure to excitation light. If the tissue expressing GFP is injured by a toxic chemical, the level of fluorescence changes due to the leakage of GFP into the hemolymph. GFP fluorescence has actually been detected in the hemolymph of the transgenic silkworm after the injection of tissue-injuring chemicals such as CCl_4 and salicylic acid (*25*). However, the observation of changes in the intensity of GFP fluorescence from outside the silkworm was hampered by the presence of uric acid. An attempt was made to produce a transparent silkworm to overcome this problem. A previous study by Tamura *et al*. indicated that the body surface of silkworms became translucent upon the intake of allopurinol, an inhibitor of uric acid synthesis, or melamine, an inhibitor of uptake of the uric acid into epithelial cells (*26*). The transgenic silkworm also became transparent when fed a diet containing allopurinol, melamine, and citric acid (*25*). When the transparent transgenic silkworm was exposed to excitation light, GFP fluorescence was clearly observed in the tissue of the fat body (*25*). Furthermore, the intensity of GFP fluorescence in the silkworm decreased with injection of tissue-injuring chemicals (*25*). This decrease occurred in a time- and dose-dependent manner (*25*). These results suggest that the transgenic silkworm expressing GFP can be used to continuously evaluate drug-induced tissue injury.

As described above, the silkworm can be effectively used as an alternative invertebrate model for evaluation of chemical toxicity, infectivity, innate immunity, and virulence (*13,27-29*). The therapeutic effects of antibiotics and anti-hyperglycemic drugs can also be analyzed using silkworm models (*11,30,31*). The present article has described the use of silkworms to evaluate drug-induced tissue injury (Figure 1). The transparent transgenic silkworm expressing GFP may be the first model that allows continuous observation of drug-induced tissue injury *via* a non-invasive means. The use of silkworms will help to reduce the number of mammals sacrificed and streamline drug development.

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