3D Skin Comet Assay

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38.1 Introduction

The comet assay allows the investigation of DNA damage in any cell type or tissue, which can be subjected to single cell isolation as it does not generally rely on proliferating cells. Therefore, the method is widely used in different areas like ecological and human monitoring or the analysis of DNA damage and repair. In addition, its versatile use in *in vivo* testing has been acknowledged to monitor effects of the first site of contact as well as organ-specific downstream effects, which are documented by the comet assay as DNA damage that may lead to clastogenic lesions or gene mutations. Recently, its increased recognition for regulatory testing led to the implementation of the *In Vivo* Mammalian Alkaline Comet Assay OECD Testing Guideline (OECD TG 489) [1].

Among the variety of *in vitro* models being subjected to the comet assay, several dermal test systems have been used to address the skin as the first site of contact for cosmetic ingredients, agrochemicals, and a growing number of pharmaceuticals. The following compilation concentrates on cell cultures of human origin, as they are of most relevance for human safety assessment. The first approaches utilized several 2D monolayer cultures, namely, primary keratinocytes (e.g., [2]), fibroblast (e.g., [3]), and melanocytes (e.g., [4]) or the HaCaT keratinocyte cell line (e.g. [5, 6]). The first study in which test compounds were applied on top of a 3D reconstructed skin model, namely, EpiSkin™ (SkinEthic™, France), was published in 2006 [7]. DNA damage was, however, not evaluated in the skin cells but in dendritic cells cultured in the medium below the skin model. Another approach published by Reus et al. [8] focused

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on the investigation of keratinocytes from *ex vivo* human skin. Twenty known genotoxins and non-genotoxins were applied atop of punch biopsies and were all predicted correctly.

Starting in 2007, a joint research project focused on a commercially available epidermal skin model, EpiDerm™ (MatTek, MA). After protocol transfer and optimization, three laboratories tested five coded genotoxic and non-genotoxic chemicals each by exposing the tissues from the top. They achieved a very high predictivity of >90% when considering the final call for a study, which comprised three independent experiments per chemical [9]. However, a relevant number of experiments had to be classified as invalid due to high values in the negative and solvent controls, accompanied by a suboptimal reproducibility. In consequence, efforts were undertaken to evaluate the suitability of commercially available full-thickness models, which consist of an epidermis and an underlying dermis connected by a basal membrane. It could be shown that the EpiDerm™ Full Thickness (EpiDerm™ FT; MatTek, MA, Fig. 38.1a) and the Phenion® Full-Thickness Skin Models (Phenion® FT; Henkel, Germany, Fig. 38.1b) were more reliable compared to the epidermal model initially used [10]. In a joint project comprising five European and US-American laboratories, EpiDerm™ FT and the Phenion® FT demonstrated lower and more consistent levels of background DNA damage in the negative and solvent controls as well as a dose-dependent increase in DNA migration after exposure with

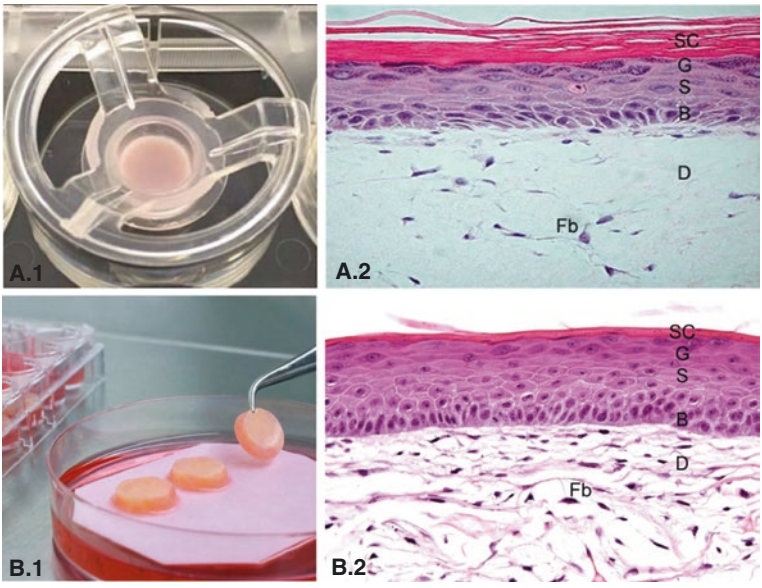

Fig. 38.1 Representative macroscopic views (A.1 and B.1) and cross sections (A.2 and B.2) of the EpiDerm™ FT (**a**) and the Phenion® Full-Thickness Skin Model (**b**). Hematoxylin and eosin-stained paraffin sections of both tissues reveal a fully differentiated epidermis containing distinct basal (B), spinous (S), granular layers (G), and a well-developed stratum corneum (SC). The collagen-based dermis (D) contains numerous primary dermal fibroblasts (Fb) (400X)

a positive control [10]. Furthermore, the rate of invalid experiments was negligible. In summary, this approach provides all advantages, which are linked with the use of 3D human reconstructed skin tissues, and adds the benefits of offering the possibility to assess the dermis as a second organ compartment. The tissues allow for topical application of compounds to mirror bioavailability relevant for the situation of use which is determined by the barrier function of the skin, mediated by the stratum corneum in the first instance (Fig. 38.1), and the organ- and species-specific xenobiotic metabolism. Furthermore, the skin models consist of p53 competent primary cells of human origin presuming normal cell cycle control. 3D tissues in general reflect cell-cell or cell-matrix interactions, as seen *in vivo*, more appropriately. The dermis, as an additional organ compartment, facilitates not only an intensive cross talk, pivotally contributing to the phenotype of the epidermis but also adds to the metabolic competency of the skin models [11]. Specifically, it could be shown that fibroblasts and keratinocytes cultured in 3D show a clear increased metabolic capacity compared to cells from the same donor propagated as 2D monolayer culture [11].

The prioritized tissues are commercially available in contrast to fresh *ex vivo* human skin, which in theory can also be used; however, it would be more difficult to handle and is not accessible globally. In consequence, the activities regarding the 3D Skin Comet assay, as further detailed below, concentrate on full-thickness skin models.

38.2 Principle of the Test Method and Scientific Basis

The comet assay methodology has first been introduced as single cell gel electrophoresis assay by Östling and Johanson [12]. Separated cells were imbedded in micro agarose gels. The cells were subsequently lysed with detergents under high salt conditions to degrade cellular and nuclear membranes and to liberate proteins like histones in order to prepare the DNA for subsequent electrophoresis. The method was further developed by Singh et al. [13] who introduced high alkaline conditions (pH > 13) during electrophoresis and a prior unwinding step, which allowed the detection of a broader range of DNA damage. After electrophoresis, which separates DNA according to size, the DNA is stained with an appropriate fluorescent dye to prepare for analysis with a full- or semiautomated image

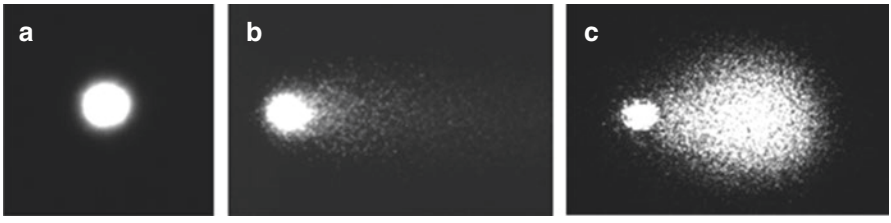

Fig. 38.2 A picture of (**a**) a comet representing normal non-fragmented DNA, which remains in the position of the nuclear DNA under the chosen electrophoresis conditions, while damaged DNA migrates toward the anode forming an increasing comet "tail" (**b**) and (**c**)

analyzer. The resulting structures appear in a comet-like shape with a head consisting of intact DNA (Fig. 38.2), which was not able to migrate under the used electrophoresis conditions due to its size, and a tail consisting of migrated DNA fragments and relaxed DNA loops, which appear after manifestation of strand breaks.

The high alkali condition allows for the detection of DNA double-strand breaks or single-strand breaks which may result from direct interaction of the test compound with the DNA or which are related to incomplete excision repair and alkali labile sites (OECD TG 489 [1]). In consequence, the modification by Singh enabled not only the detection of clastogenic DNA damage but also the identification of lesions which could be precursors of gene mutation events.

There are several parameters that are used to measure the extent of DNA that has migrated during electrophoresis and the distance it has traveled. Among these, the fluorescence intensity in the comet tail in comparison to the head (% tail DNA or % tail intensity) is of specific interest because it is considered linearly related to the DNA break frequency over a wide range of DNA damage [14]. This parameter has been recommended to assess DNA damage by the OECD TG 489 [1] and is also used for the methodology described here.

The comet assay in general is considered an indicator test since the DNA damage detected could be repaired or may be lethal to the cell resulting in nonpersistent effects. However, the strand breaks could also be fixed into mutations or chromosomal damage both resulting in permanent DNA damage of viable cells. In a recently published analysis of rodent carcinogens giving negative or equivocal results in the *in vivo* micronucleus test, the *in vivo* comet assay was positive for approximately 90% of these chemicals and was negative for nearly 80% of the non-carcinogens. Thus, the *in vivo* comet assay revealed a better predictivity in comparison to the *in vivo* rodent transgenic mutation assay or the *in vivo* UDS (Unscheduled DNA Synthesis Assay), [15]. Meanwhile the alkaline version of the comet assay, as described here, is the most widely used comet assay protocol, which has also been recommended for genotoxicity assessment by the International Workshop on Genotoxicity Testing (IWGT, formerly IWGPT; [16]).

38.3 Current Validation Status

3D skin assays have been flagged early for their potential to follow-up on positive results from *in vitro* genotoxicity assays, and consequently validation efforts have been suggested [17, 18]. Five European and US-American laboratories have since evaluated the within and between laboratory reproducibility of the 3D Skin Comet assay using full-thickness skin models. The generation of information on predictivity of the assay is in progress as 30 compounds, selected by external experts, are being investigated. The chemicals cover a balanced set of true positive and true negative chemicals, which showed concordant results in historical *in vitro* and *in vivo* testing, as well as a subset of compounds, the so-called irrelevant positives, for which positive results were obtained *in vitro* that did not correlate with historical *in*

vivo genotoxicity or carcinogenicity studies. In addition, the chemicals represent different chemical classes and modes of action. For each of the selected chemicals, high-quality *in vitro* and *in vivo* data are available. However, only chemicals for which data from dermal *in vivo* studies exist can be used. This group of chemicals is limited and can therefore only support one standardized study. The ongoing ring trial is considering the respective validation standards; e.g., chemicals are tested double blinded, i.e., each chemical has an individual code, which differs between laboratories, though testing the same compound.

The ongoing study focuses on the investigation of the Phenion® FT using a so-called lean design. In the first phase, eight compounds were investigated by three laboratories. The data analysis showed that the reproducibility within and between the participating laboratories was sufficient to test each of the remaining 22 chemicals only in one laboratory during a second and last phase [10]. The validation will be finalized in 2017.

38.4 Performance and Applicability of the Test Method

In general, 3D skin models support testing of a great variety of compounds and compensate certain downsides of submerged 2D monolayer cultures. They allow for testing of lipophilic compounds and for application of higher concentrations if relevant for the situation of use. In addition, they facilitate testing of particulate materials although this has to be approached with caution as described below for the occurrence of precipitation.

Acetone or 70% ethanol (v/v) are used during the validation exercise. While tranfering the assay to other laboratories it has to be proven that these or other solvents do not disturb the air-liquid interface which is essential for a proper tissue cultivation over a period of 48 h [9]. Extensive precipitation of solids as well as small droplet of lipophilic liquids should be avoided as they may also disturb the air-liquid interface with the potential risk of causing false-positive results.

Taken these prerequisites into consideration, the results available from phase I of the validation indicate good reproducibility and predictivity comprising data of a pro-mutagen, a cross-linker, two direct-acting mutagens, as well as four compounds with an expected negative outcome. Colored substances were tested in parallel to the validation and did neither interfere with the tissue's integrity in the cultivation phase nor with DNA evaluation [19, 20].

Furthermore, skin models are increasingly used to investigate the impact of UV light on skin, which can directly modify DNA causing the formation of pyrimidine dimers as the major effect [21]. In consequence, 3D skin tissues have been used for the assessment of photoprotective compounds like UV filters [21]. For the general assessment of photogenotoxic effects, an epidermal model has successfully been used to assess the impact of UV light on DNA integrity with the comet assay [7]. After EpiSkin™ tissues were irradiated with UVA or solar-simulated light, keratinocytes were analyzed for DNA migration as a proof of concept for this approach. The use of 3D skin tissues is a relevant step forward in comparison to submerged 2D monolayers cultures. However, it should be noted that these nonstandard

photo-genotoxicity approaches have not yet been investigated with regard to their predictive capacity and reproducibility within or between laboratories.

38.5 Brief Description of the Protocol

38.5.1 Study Design

The investigation of a specific compound with the 3D Skin Comet assay comprises a certain set of experiments similar to studies of standard *in vitro* genotoxicity assays (Fig. 38.3a). (1) First, an appropriate solvent is selected to dissolve the chemical before exposing the tissue, targeting a maximum concentration of 10 mg/100 µL or 10%, respectively. (2) The dose-range-finding experiment is designed to narrow down the dose range and especially to enable a decision on the maximum use concentration which could be limited by (a) the limit dose previously mentioned (10 mg/100 µL), (b) cytotoxicity, or (c) solubility/precipitation of the test compound. Cytotoxic effects of the test compound are measured in the form of intracellular adenosine triphosphate (ATP) concentration [22] and activity of adenylate kinase, which is released from the cells into the culture medium upon cell damage [23]. (3.1) Verification of a clear positive finding will usually not be required;

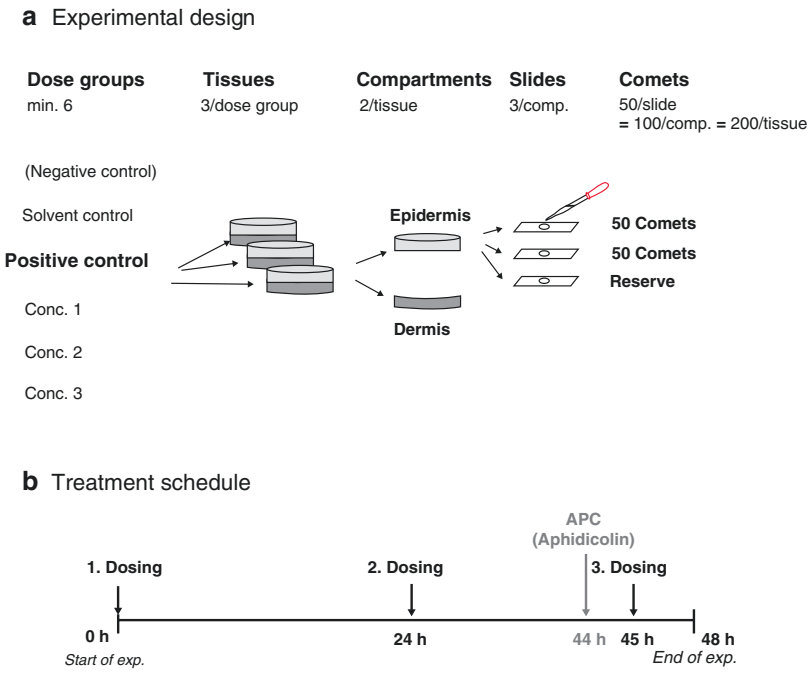

Fig. 38.3 Schematic of (**a**) the experimental design and (**b**) the treatment schedule. For details, please refer to Sect. 47.5. *comp.* compartment, *min* minimum, *exp.* experiment

however, a confirmatory second experiment should be added during the validation process. (3.2) In case the test item provides negative or inconclusive results, an additional test run should be performed using aphidicolin (APC), which is added 4 h before the end of the treatment period (Fig. 38.3b). APC, an inhibitor of DNA repair processes, was introduced into the protocol to improve the assay's sensitivity by accumulating excision repair-related strand breaks. This specific approach, which is outlined in paragraph 38.5.2 in more detail, has been shown to better reflect the assessment of pro-mutagens [24]. In the experiments complemented by APC, the pro-mutagen benzo(a)pyrene is used as positive control instead of methyl methane sulfonate (MMS) to prove the effectiveness of APC. (3.3) In case of non-concordant or equivocal findings in the APC experiment, a third test run with modified (usually tighter) concentration spacing is recommended.

38.5.2 Experimental Design

At least three concentrations of a test compound should be investigated in a test run complemented by a solvent control and a positive control (MMS, a direct-acting mutagen) group. The tissues are treated 48 h in total to ensure possible metabolic processing of the compound (see Fig. 38.3b). Twenty-four hours and 45 h after the first dosing, a second and third aliquot of the test compound is applied atop of the same tissue. Especially the latter time point is intended to capture damage, which may be subject to immediate DNA repair. To note: when establishing the assay, both negative (untreated) and solvent control groups should always be included. Once sufficient solvent control data is available which indicates that the solvent control has no impact on the background DNA damage of the tissues, untreated skin models do not need to be added anymore.

38.5.3 Cell Isolation and Comet Assay Procedure

At the end of the exposure period of 48 h, keratinocytes and fibroblasts are isolated in a tissue-specific procedure. The two compartments of the EpiDerm™ FT are separated using forceps before keratinocytes and fibroblasts are isolated separately by successive treatments with PBS, EDTA, and trypsin. The Phenion® FT is first incubated in thermolysin to allow for the degradation of the basal membrane and the subsequent separation of epidermis and dermis. Afterwards both cell types are isolated mechanically using a mincing procedure, similar to protocols used for the *in vivo* comet assay, leaving a mixture of cells and free nuclei. Subsequently, cells from both tissues are subjected to the same comet assay procedure in which cells/nuclei are first resuspended in low melting agarose (0.5%) which is then transferred onto glass slides. They are subjected to a lysis procedure overnight, which degrades cell and nuclear membranes by exposure to detergents. A high salt concentration removes proteins like histones. Afterwards, DNA strands are separated by high alkali conditions (pH >13) before DNA migrates to the anode during 30 min of

electrophoresis using fresh high alkali buffer. Finally, the slides are neutralized and dried.

38.5.4 Analysis

After the experiment, four slides per skin model are subjected to analysis (two each for epidermis or dermis) as the evaluation of two slides per compartment and 50 comets per slide (i.e., 100 comets per cell type) was considered sufficient [25] (Fig. 38.3a). Before analysis, slides are randomized and stained with an appropriate fluorescence dye (e.g., SYBR Gold). The fluorescence intensity in the comet tail compared to the respective comet head is afterward analyzed semiautomated using a 200x magnification of a fluorescence microscope and comet assay image analysis software (sold by a variety of commercial providers, freeware is also available).

The 100 data points (2 × 50 comet measures/slide) per skin compartment (epidermis or dermis) are subjected to a variance-stabilizing transformation before they are summarized as median. Since each control or dose group is represented by three tissues, three medians are finally summarized as mean value for each dose or control group. These mean values are used for further statistical analysis since the skin tissue is considered the experimental unit.

Before evaluating the results for genotoxicity, the validity of an experiment is determined applying defined thresholds for % tail intensity for solvent and positive controls. Furthermore, the validity of a dose group is evaluated applying thresholds set for the two cytotoxicity measurements, which have been identified for the 3D Skin Comet assay, i.e., the intracellular concentration of ATP and the activity of adenylate kinase released into the culture medium. The latter criteria were established because DNA damage can be triggered by cellular toxicity, e.g., when cells go into apoptosis or necrosis. Therefore, as it is the case for other genotoxicity assays, increased DNA damage that occurs only in conjunction with strong cytotoxicity is not considered as biological relevant in the context of a genotoxicity assessment.

In the next step, an analysis of variance (ANOVA) of the solvent control and the dose groups is conducted. In case of a positive ANOVA, the statistical significance of an effect is analyzed by a pairwise comparison of the solvent control and single-dose groups using the Dunnett test. A test substance is considered to be genotoxic in the 3D Skin Comet assay if one or more concentrations produce a statistically significant increase in the percentage of tail DNA at concentrations that do not exceed the limits of cytotoxicity (for details, see [10]). In addition to the statistical evaluation, the following criteria, which are in line with procedures established for OECD TG 489 [1], need to be fulfilled to consider the biological relevance of effects observed. In brief, (a) the response has to be dose dependent, (b) at least one dose needs to be statistically significant different from the solvent control, and (c) at least one test group needs to be outside the historical control data range. If none of the three criteria is fulfilled, the test item is called negative. In case one or two but not all the criteria for a positive call are fulfilled, the test compound is considered

negative, or equivocal, and further testing may considered (see also paragraph 38.5.1 on study design).

38.6 Perspectives from the Test Developer

38.6.1 Critical Steps in the Protocol

As with all variants of the comet assay, certain steps of the protocol need specific attention to support a high level of standardization and reproducibility within a laboratory. Altering sample preparation, electrophoresis conditions or microscope settings have been investigated and showed an effect on the DNA migration measured (e.g. [26]). These points have also been taken into account for the 3D Skin Comet assay protocol and are applied in the ongoing validation exercise. For example, trainings have been organized before the validation to ensure a proper implementation of the cell isolation procedures in the participating laboratories to avoid induced DNA damage caused by cell separation. To minimize between laboratory variability, the electrophoresis conditions were standardized by the use of a common commercially available electrophoresis chamber, uniform electrophoresis time, as well as voltage settings. In addition, standards for the analysis of slides have been agreed on and were published recently [9].

As with all other methods, laboratories should establish experimental competency in the 3D Skin Comet assay. Proof of competency could include a series of experiments providing low and reproducible % tail DNA values in non-treated or solvent exposed tissues. During the course of this proficiency phase, the laboratory should build a historical database of the solvent and negative controls. The same applies for the positive control using concentrations which induce a range of DNA damage that spans slight to clear DNA damage.

38.6.2 Possible Protocol Adaptations

The protocols for cell isolation and the comet assay procedure have been optimized and should provide high-quality data. However, the design of an experiment or an entire study might be optimized/modified further after evaluation of the entire data set generated in the ongoing validation exercise. A possible point for optimization could be the focus on one cell type to improve the throughput of the method. Protocol adaptations made after the first phase of the validation exercise included the rule that a negative control of untreated tissues will only be needed in case none of the two recommended solvents is used. This was decided after sufficient data became available showing that solvent and untreated controls did not differ in terms of their background DNA damage.

Apart from such data-driven changes, the standard protocol can be amended to gain mechanistic insights into DNA damage or to better reflect DNA damage induced by agents which cause cross-linking of DNA with DNA or protein. Such damage

cannot be detected reliably with the standard experimental design [27]. Mitomycin C (MMC), as an example of a DNA-DNA cross-linker, generates covalent bonds between guanine nucleotides. These links between two DNA strands do not only suppress positive comet signals at higher doses but can even lead to a reduction of measurable strand breaks if compared to control. Therefore, a modified protocol that was developed for efficient detection of cross-linkers [28] was adapted to the Phenion® FT. Using this protocol, tissues were not only exposed to MMC but were co-treated with MMS to generate a high background level of single-strand breaks that allowed for efficient detection of a reduction in measurable DNA breaks [10].

The alkaline version of the comet assay not only enables the detection of strand breaks which are formed as direct effects of a treatment, it also allows detection of strand breaks which evolve in the course of excision repair processes in response to UV radiation, alkylating agents, or bulky adduct formation, to name a few. These strand breaks, set by specific enzymes to remove modified nucleotides or bases, can be short-lived. The incorporation of enzyme inhibitors to the comet assay protocol supports the accumulation of these DNA repair-related strand breaks thereby amplifying comet formation and increasing the sensitivity of the assay [29]. Two groups of inhibitors have successfully been included into comet assay protocols. The first group of inhibitors interferes with DNA repair and replication in general and contains, e.g., hydroxyurea (HU), which inhibits ribonucleotide reductase causing an imbalance in the nucleotide pool [30]. Cytosine arabinoside (ara-C) is incorporated into DNA during replication, after it was converted to cytosine arabinoside triphosphate, leading to chain termination and cell cycle arrest [31], and aphidicolin (APC) which was showing to inhibit DNA polymerases α and δ [29]. The latter one has been included in the 3D Skin Comet assay protocol, in case of negative findings in the first main experiment. APC is added 4 h before the experiment is terminated (44 h after the first treatment) (Fig. 38.3b). While strand breaks increase due to excision repair processes, the impact of this DNA repair enzyme inhibitor on cell proliferation is negligible. APC is added only for a limited period of time in which the keratinocytes of the stratum basale and stratum spinosum, the only proliferating cells in the tissues, are not affected due their low turnover compared to monolayer cultures. The marginal increase of % tail DNA in the solvent control after adding APC proved the suitability of the skin tissues for this approach which has been shown to better reflect the assessment of pro-mutagens, while the predictivity of non-genotoxins remained high with 100% [10, 24].

A second group of inhibitors, i.e., lesion-specific enzymes, can also be incorporated into the 3D Skin Comet assay to characterize DNA damage. These enzymes remove modified DNA bases leaving an apyrimidinic or apurinic (AP) site, which is subsequently converted to a single-strand break under high alkali conditions. In theory, any lesion for which a specific enzyme exists can be detected in this way. Hitherto, several enzymes, like 8-oxo-Gua DNA glycosylase (OGG1), have been identified to investigate oxidative DNA damage [32]. Alkylated nucleotides like 3-methyladenine can be identified by 3-methyladenine DNA gycosylase II (AlkA) [33], while uracil DNA glycosylase (UDG) has been shown to support the detection of uracil, as a miss-incorporated DNA base [34]. Furthermore, bulky adducts can be

identified by applying uvrABC, an exonuclease derived from *E. coli* [35], whereas the enzyme T4 endonuclease V helps in detecting the dimerization of adjacent pyrimidine dimers which are observed as characteristic lesions induced by UV light [36]. The use of these lesion-specific enzymes has added value to the comet methodology in general and may add to the 3D Skin model approach in the future.

38.7 Conclusions

The 3D Skin Comet assay, together with the RSMN (Chap. 46), is considered to close a gap in the toolbox of *in vitro* genotoxicity assays since they have been shown to be advantageous for the evaluation of dermally exposed substances. The full-thickness models consist of human primary p53 competent keratinocytes and fibroblasts differentiating this approach from many *in vitro* genotoxicity assays, which are based on rodent cancer cell lines, some of them being p53 deficient. Furthermore the cultivation of keratinocytes and fibroblasts in a three dimensional environment not only supports the *in vivo*-like phenotype of the cells but also enables the topical application of compounds on top of the stratum corneum close to the situation of use. A validation study is ongoing, and the results generated so far indicate good reproducibility and predictivity of this method.

In parallel to the validation, the 3D Skin Comet assay has already been used to follow-up on unfavorable results from the standard genotoxicity *in vitro* test battery for regulatory testing of cosmetic ingredients [19, 20]. Successful validation is hoped to lead to wider regulatory acceptance that will include more product categories for which the dermal route is relevant for risk assessment.

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