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Expression of antimicrobial peptides by uveal and cutaneous melanoma cells and investigation of their role in tumor cell migration and vasculogenic mimicry

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Abstract

Aims: Antimicrobial peptides (AMPs) have been implicated in the pathogenesis of several cancers, although there is also evidence suggesting potential for novel, AMP-based anti-tumour therapies. Discerning potential roles of AMPs in tumour pathogenesis may provide valuable insight on mechanisms of novel AMP-based anti-tumour therapy.

Methods: mRNA expression of the AMPs α defensin (*HNP-1*); cathelicidin (*LL-37*); and β defensins (*hBD-1*, *hBD-2*, *hBD-3*, *hBD-4*) in human uveal and cutaneous melanoma cell lines, primary human uveal melanocytes, and primary human uveal melanoma cells was determined by RT-PCR. An in vitro scratch assay and custom Matlab analysis were used to determine AMP effects on melanoma cell migration. Lastly, the effect of specific AMPs on vasculogenic mimicry was determined by 3D culture and light and fluorescence microscopy.

Results: Low to moderate, AMP transcript levels were detected and these varied across the cells tested. Overall, *LL-37* expression was increased while *hBD-4* was decreased in most melanoma cell lines, compared to primary cultured uveal melanocytes. There was no observable influence of *HNP-1* and *LL-37* on tumour cell migration. Additionally, aggressive cutaneous melanoma cells grown in 3D cultures exhibited vasculogenic mimicry, although AMP exposure did not alter this process.

Conclusions: Collectively, our data show that although AMP mRNA expression is variable between uveal and cutaneous melanoma cells, these peptides have little influence on major characteristics that contribute to tumour aggressiveness and progression.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Antimicrobial peptides (AMPs) are small, cationic, peptides that function as antimicrobial agents and immune modulators.[1] Compared to normal tissues, AMPs are differentially expressed by many cancers, such as lung, colon, kidney and breast, implying a pathogenic role. Recent studies support AMP modulation of tumour characteristics, such as proliferation, migration, invasiveness, and neovascularization.[2–3] Some AMPs have anti-tumour properties via a direct cytolytic effect or the induction of tumour cell death, as well as chemotaxis, and activation of immune cells, which mount tumour-related inflammatory responses.[4–5] Therefore, depending on tumour type and/or microenvironment, AMPs may positively or negatively impact the development of cancers.

Despite studies in a wide range of cancers, the expression profile of AMPs in uveal melanoma, the most common primary ocular tumour in adults,[6] has not previously been investigated, and the effects of AMPs on behaviour of these cells are unknown. Further, few studies have addressed AMPs in cutaneous melanomas, although it has been reported that human β -defensin (hBD)-2 has suppressive effects on cutaneous melanoma cell activities such as proliferation, whereas the effects of the cathelicidin LL-37 are stimulatory.[7–8] Notably, AMPs have been implicated in stimulating tumour cell migration, for example LL-37 promoted metastasis in breast cancer,[10] and migration and invasion of melanoma cells.[7] Therefore, in the present study, we addressed some of these gaps in the literature. For cell behaviour, we focused on migration, which is an important property of most malignant cells, and vasculogenic mimicry, which is seen in many aggressive tumours.[9]

Vasculogenic mimicry is a pro-tumour process involving formation of fluid-conducting channels by highly invasive, genetically dysregulated tumour cells.[9, 11,] These channels, notably devoid of endothelial cell involvement, anastomose with blood vessels, thereby improving nutrient delivery into the tumour, and thus are an important regulator of growth. [12] Vasculogenic mimicry is typical of more aggressive melanoma phenotypes,[13] and consequently, patients exhibiting it have a poorer prognosis.[14] The detailed mechanisms underlying vasculogenic mimicry formation are still being elucidated, but it is associated with cancer cells with altered extracellular matrix gene expression that proliferate, migrate and organize into patent channels in response to angiogenesis promoting factors and the tumor microenvironment. [15] In addition to stimulating migration of some tumour cells, AMPs such as LL-37, PR39 and α defensins, can promote blood vessel development[16–17] and interfere with tumor-associated angiogenesis.[4,18] Thus, based on the findings in other tumour types, we hypothesized that AMPs may influence melanoma cell migration and vasculogenic mimicry formation.

MATERIALS AND METHODS

Tumour Cells

OCM8 and OMM2.5 cells were provided by Dr. Jerry Y. Niederkorn of UT Southwestern, Dallas, TX, and were originally donated by Dr. June Kan-Mitchell from the University of California, San Diego, CA, and Dr. Bruce Ksander from Schepens Eye Research Institute, Boston, MA. SP6.5 cells were provided by Dr. Miguel N. Burnier from the Henry C.

Witelson Ocular Pathology Lab in Montreal, Canada. MUM2b cells were provided by Dr. Arthur S. Polans from the Department of Ophthalmology and Vision Science of the University of Wisconsin. Samples of RNA extracted from human primary uveal melanoma and normal uveal melanocytes were generously provided by Dr. J. William Harbour of the University of Miami, FL., and were collected and handled with consent, under approval of the University of Miami Institutional Review Board.[19] Melanoma cell lines were maintained in culture media containing penicillin/streptomycin, and amphotericin B (Life Technologies, Grand Island, NY) as follows: OCM8 and OMM2.5 RPMI-1640 with 10% fetal bovine serum (FBS); SP6.5 RPMI-1640 with 5% FBS; MUM2b DMEM with 10% FBS. All cells were cultured in 5% CO₂ at 37°C. Short Tandem Repeat (STR) analysis (University of Arizona Genetics Core, Tucson, AZ) was performed on genomic DNA to authenticate the cell lines.

AMP Expression by Reverse Transcription PCR

RNA was extracted using RNeasy Mini kits (Qiagen, Valencia, CA). Reverse transcription was carried out using a first strand cDNA synthesis kit (BioChain Optimax, Newark, CA) with random hexamers, and PCR was performed with Brilliant III Ultrafast SYBR green QPCR master mix (Agilent, La Jolla, CA) using custom designed, and previously published primers for *hBD-1*, *-2*, *-3*, *-4*, *LL-37* and *HNP-1* that were sequenced to confirm the product.[20–23] *RPL27* was used as a reference gene based on its small coefficient of variation and small maximum fold change in expression across tissue types and experimental conditions.[24] Primer sequences are shown in Supplemental Table 1. PCR was performed using a Stratagene MX3005P as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 20 seconds, with appropriate no template and no RT controls, and products were ran on a gel to confirm the right sized targets. Tissue control RNA (BioChain), run in parallel, included skin for *hBD-1*, *-2* and *-3*, testis for *hBD-4*, and bone marrow for *LL-37* and *HNP-1* (Supplemental Table 2). Quantitative real time PCR data were analyzed using Relative Expression Software Tool – Multiple Condition Solver version 2 (© 2005–2006 Pfaffl MW, Horgan GW, Vainshtein Y, Avery P)[25] Expression ratios, relative to primary uveal melanocytes, were tested for significance by a pair-wise, fixed reallocation randomisation test, and plotted using standard error (SE) estimation using Taylor's series.

In Vitro Scratch Assay

Melanoma cells were seeded in 6 well plates and grown to confluence. Two parallel lines running across each well were made on the underside of the plate with a black pen as reference points for digital imaging. Cells were serum starved for 6 hours, the media was removed and replaced with PBS, and a sterile 200µl pipette tip used to make 3 parallel scratches perpendicular to the reference lines. The PBS was then aspirated and replaced with serum-free media containing 5 or 17.5µg/ml HNP-1, 5 or 20µg/ml LL-37 (American Peptide Company, Sunnyvale, CA), or 10% FBS in culture media. Serum-free media acted as a negative control.

To assess wound closure, images were captured using an Olympus IX71 Inverted Microscope (Center Valley, PA) equipped with a Qimaging Rolera-XR Fast 1394 digital

imaging system (Surrey, BC) at 0, 6, 12, 24, and 48 hours post-scratch. The images were analyzed using a custom Matlab program that determined the average width of the cell free area. The measurements were normalized to the width of the cell free area at time 0, which was set to 100%. Based on published data using this methodology, three independent experiments were performed and the data were analyzed by One-way ANOVA with Dunnett multiple comparison test on GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, California). Data were compensated using a Greenhouse-Geisser correction for equality of variance.

3D Cell Cultures and Imaging of Vasculogenic Mimicry

Matrigel (BD Biosciences, San Jose, CA) was transferred to each well (100µl/well) of an 8-chamber glass cover slip slide. The slide was incubated at 37°C for 30 minutes to polymerize the gel and MUM2b cells (1×10^5) were seeded on top. Growth media with 10% FBS, containing either HNP-1 (12.5 or 17.5µg/ml) or LL-37 (5 or 10µg/ml) were added to individual wells with 100% media changes every 3 days.

For imaging, the Matrigel blocks were fixed in 2% paraformaldehyde for 1 hour at room temperature, washed with warmed (37°C) PBS, permeabilized with 0.1% Triton-X for 30 minutes at room temperature, then rewashed. The blocks were stained by adding equal volumes of DAPI (Molecular probes, Eugene, OR), and either Phalloidin (Molecular Probes) or Calcein AM (Life Technologies), and incubating for 45 minutes at room temperature. Bright field and fluorescence images were taken every 3 days for up to 21 days to identify formation of looping patterns characteristic of vasculogenic mimicry.

RESULTS

Cell Authentication

STR analysis confirmed the OCM 8 and OMM 2.5 profiles to be identical to previously published data, [26] hence they were confirmed as uveal melanoma cells. MUM2b matched previously published analyses [26] for cell line C918, most recently confirmed to be C8161, a cutaneous melanoma cell line. [27] SP6.5 matched IRG-1 cells, also a cutaneous melanoma line.

Melanoma Cell AMP Expression

AMP expression in uveal melanoma cell lines, (OCM8, OMM2.5), cutaneous melanoma (MUM2b, SP6.5), pooled data from three primary cultures of human uveal melanomas (UM 110P, UM 10A, UM 146), and pooled data from two primary cultures of uveal melanocytes (UM 07-1A, UM 07-2CB) are presented in Figure 1. Expression levels were classified as moderate ($C_q < 30$) low ($C_q 30-35$), or not expressed ($C_q > 35.5$). There was low to moderate, but variable expression of AMPs across the cells tested (Supplemental Table 3), with moderate (*hBD-4*) to low expression (*hBD-1*, *hBD-2*, *HBD-3*, *LL-37*, *HNP-1*) in both primary uveal melanocytes and primary uveal melanoma cells, with no significant differences between these two cell types. Compared to primary uveal melanocytes, *hBD-4* and *HNP-1* expression was significantly down-regulated in the uveal melanoma cell lines OCM8 and OMM2.5, with *HNP-1* expression below the threshold of detection in OCM8

cells (Figure 1). In OMM2.5 cells only, *hBD-1* decreased to undetectable levels and *LL-37* was significantly increased. In both OCM8 and OMM2.5, *hBD-2* and *hBD-3* expression levels were comparable to primary cultured melanocytes. In both the cutaneous melanoma cell lines (MUM2b and SP6.5), *LL-37* was significantly up-regulated, compared to primary melanocytes, whereas *hBD-4* was significantly down-regulated only in MUM2b cells. There were more AMPs with significantly altered expression in SP6.5 cells, with *hBD-1* and *hBD-2* significantly up-regulated and HNP-1 significantly down-regulated. Overall, none of the AMPs analyzed showed identical patterns of gene expression changes in all 4 melanoma cell lines, compared to normal uveal melanocytes. However, there were some parallels, *hBD-4* expression down-regulated in OCM8, OMM2.5 and MUM2b, and *LL-37* expression up-regulated in OMM2.5, MUM2b and SP6.5.

Effect of AMPs on Melanoma Cell Migration

Figure 2A shows a monolayer of untreated OCM8 cells at various times after a scratch wound was created. At 0 hours, there was a clean, well-defined wound edge. By 12 hours after the scratch (or possibly earlier), cell migration was detected as an irregular wound edge. At 24 hours, more cells populated the denuded area, and, by 48 hours, the wound edge became increasingly irregular and in many regions the monolayer was re-established across the wound. Treatment of OCM8 cells with either HNP-1 (17.5µg/ml) or *LL-37* (5µg/ml) did not affect wound closure (Figure 2B, right panel).

As shown in Figure 2B (left panel), scratch wounds in SP6.5 cutaneous melanoma cells treated with media containing 10% FBS showed an increased closure rate, such that at 48 hours the wound was nearly 50% closed, compared to approximately 15% closed in the serum-free group. Treatment of SP6.5 cells with HNP-1 at 17.5µg/ml caused the wound to close faster compared to the serum free control, although this did not reach statistical significance ($p = 0.0752$, ANOVA).

Effect of AMPs on Vasculogenic Mimicry

The highly aggressive MUM2b cells grown in a 3D matrix for 3 days formed looping patterns, with cells arranged around hollow cores, characteristic of vasculogenic mimicry (Figure 3A). At higher magnification (Figure 3B), cellular packing around empty loops can be seen forming the basic framework for subsequent channel formation. After 17 days (Figure 3C), cells populated the insides of the once empty looping patterns, with the edges forming the framework for subsequent channel formation. As expected, the less aggressive, OCM8 cells did not form looping patterns or networks (Fig. 3D).

Fluorescence microscopy was used to further characterize the patterns formed by MUM2b cells in 3D culture. Figure 4A shows two adjacent loops running across the field of view, and a channel with a dilated chiasm at the edge where the two loops meet (arrow). Higher magnification (Figure 4B) affords a better appreciation of the uniformity of the walls and patency of this channel. Figure 4C shows a channel looping around an aggregate of cells, and anastomoses with a larger segment of the network.

Figure 5 shows a comparison of MUM2b cells with and without AMP treatment. Untreated MUM2b cells arranged to form looping patterns with cores devoid of cells as early as a few

days after plating in a 3D substrate. By the first week, the loops appeared to have increased in diameter, and the walls became thicker and more populated, with a few cells starting to populate the cores. Progressive population of the loops caused formation of cores surrounded by looping networks by the 2nd to 3rd week, with no change in loop diameters. Exposure to LL-37 caused the loops to appear much thinner at the early time point (day 3), however this did not persist (Fig. 5). Cells treated with 5µg/ml LL-37 showed dissolution of the looping patterns by the 3rd week, but this was the only apparent difference from the untreated controls.

Treatment of MUM2b cells with HNP-1 at 17.5µg/ml, resulted in an overcrowding effect and packing of cells into the loops, possibly from increased proliferation of the cells. However, loop formation was not abrogated by HNP-1. Cellular 'islands' appeared within the loops at 3 weeks and had the appearance of a densely packed aggregate of cells, structurally resembling a solid tumour.

DISCUSSION

AMPs have been implicated in tumour pathogenesis and have shown potential as novel anti-cancer agents.[2–5] However, AMPs have not been widely studied in the context of cutaneous melanomas, and have not been studied at all in relation to uveal melanoma. Here we established the differential pattern of AMP expression by uveal and cutaneous melanoma cells, and tested effects of AMPs on migration and vasculogenic mimicry formation, but found no modulation of either of these pro-tumour characteristics.

In our survey of melanoma cells, AMP expression was generally in the low to moderate range, with Cq values in the range of 27–35 with some variability between the cell lines. Compared to primary cultured melanocytes, the most consistent changes were increased *LL-37* expression in OMM2.5, MUM2b and SP6.5 cells, and decreased *hBD-4* expression in OCM8, OMM2.5 and MUM2b cells. Increased expression of endogenous *LL-37* has been implicated in the development or progression of several cancers including breast, ovarian and lung, where it can stimulate proliferation, enhance metastatic capacity, and transactivate the epidermal growth factor receptor (*EGFR*).[3] Increased expression of *LL-37* has been reported in various skin cancers, including malignant melanoma, leading to the suggestion that it may have pro-tumor properties in skin malignancies.[7] However, *LL-37* also has demonstrated antitumor effects, such as suppression of tumorigenesis in gastric cancer.[3] A role for hBD-4 in tumour pathogenesis is less well studied, but reduced *hBD-4* expression in colon cancer has been reported.[28] Increased expression of *HNP-1*, on the other hand, has been reported in colorectal cancer, renal cell carcinoma, bladder cancer cells and squamous cell carcinoma of the tongue,[29–32] In contrast, we observed down-regulation of *HNP-1* in uveal (OMM2.5) and cutaneous (SP6.5) melanoma cell lines.

In contrast to the changes in expression of some AMPs in the melanoma cell lines, there were no significant differences in AMP mRNA expression between cultured primary uveal melanoma cells and primary uveal melanocytes. This was unexpected and suggests that, in contrast to other cancers [3, 28, 32–33], AMP expression is not modulated in uveal melanoma. One possible explanation for the differences between primary melanoma and

melanoma cell lines is that modulation of AMP expression occurs later in tumorigenic progression and/or is related to specific genetic changes, and these changes had not yet occurred in the particular primary uveal melanoma cells tested. An alternative, and not mutually exclusive possibility is that changes in gene expression in the melanoma cell lines reflect adaptation to the *in vitro* environment following prolonged time in culture.[34]

In addition to contributions of altered tumor-cell AMP expression to disease pathogenesis, AMPs from other sources, such as infiltrating immune and inflammatory cells, may also influence the oncogenic process.[35–36] Therefore, we investigated AMP effects on migration and vasculogenic mimicry formation in selected uveal and cutaneous melanoma cell lines. Specifically, we studied the effects of LL-37 and HNP-1, as these AMPs showed significant changes between primary melanocytes and the melanoma cell lines in our study, and have been shown to promote tumour cell migration and invasiveness in cutaneous melanoma and bladder cancer [7,31]. However, there were no statistically significant differences in wound closure between AMP-treated and control cells. Thus, our findings suggest that AMPs LL-37 and HNP-1 play no role in migration of the melanoma cell lines, at least in a controlled environment. In contrast to our findings, Kim et al [7] reported that LL-37 stimulated migration, invasiveness, and proliferation of the A375 malignant cutaneous melanoma cell line. This suggests that there are differential responses to AMPs, depending on the specific tumour cell line under study.

Vasculogenic mimicry is a well documented feature of highly aggressive invasive melanoma phenotypes, and has been documented in MUM2b cells *in vitro*. [9, 37] We reproduced this phenomenon in 3D cultures, where aggressive MUM2b cutaneous melanoma cells developed looping patterns that became channels, whereas the weakly aggressive OCM8 uveal melanoma cells did not. Given that AMPs can modulate behaviors such as migration, proliferation and vessel formation in melanoma and breast cancer [7, 10], we hypothesized that AMPs may influence vasculogenic mimicry. However, apart from a change towards thinner loops at early timepoints, there were no major differences in the appearance or behavior of MUM2b cells with LL-37 treatment. Higher concentrations of HNP-1, (17.5µg/ml) led to packing of the loops with cells and overcrowding at later time points, possibly resulting from enhanced proliferation. This is in keeping with previous observations showing HNP-1 stimulates DNA synthesis and cell proliferation in lung epithelia *in vitro*. [38] Otherwise, there were no observable effects of HNP-1 on the ability of the MUM2b cells to exhibit vasculogenic mimicry.

A limitation of this study is that analysis was restricted to a subset of known human AMPs, specifically those that have been shown to have dysregulated expression or have been shown to play roles in tumour pathogenesis in other tissues.[10, 29–30, 32–33, 39–40] For the gene expression analysis, the limited amount of RNA available from the primary uveal melanocytes and melanomas also limited the total number of genes that we could analyze. Availability of RNA also precluded rigorous quality control to test for RNA degradation of these samples during shipping. However, our use of random primers for reverse transcription partially compensated for this as random primers not only can generate more cDNA than oligo-dT primers,[41] they can generate cDNA from degraded transcripts because they do not rely on the presence of intact poly-A tails on the 3' untranslated regions. A strong

indicator of poor prognosis in uveal melanoma is loss of an entire copy of chromosome 3 (monosomy 3). However there are only a few reports of monosomy 3 uveal melanoma cell lines in the literature with the majority of cells tested, including the OCM8 line used here being disomy 3,[42–43] and thus of potentially lower risk/less aggressive characteristics compared to primary tumour cells that exhibit loss of chromosome 3 and are associated with larger tumour size, location in the ciliary body, presence of epitheloid cells, inflammatory infiltrates and generally worse prognosis.[44] A few cell lines, such as UPMM-1, UPMM-2, UMT33 have been shown to exhibit monosomy 3 and may serve as better models in future studies to examine the role of AMPs in uveal melanoma.[43, 45]

In summary our data show expression of AMPs in uveal melanoma and cutaneous melanoma cells lines. There was decreased expression of hBD-4 and HNP-1, and enhanced expression of LL-37 in uveal and cutaneous melanoma cell lines compared to primary uveal melanocytes, but no significant change in AMP expression between the primary tumour and normal uveal melanocytes. This may be the result of the cell lines having been derived from tumour cells that were more advanced in the pathogenic process, or may be artefactual and relate to changes acquired during the actual process used to generate the cell lines. Further, the AMPs LL-37 and HNP-1 do not significantly influence melanoma cell migration or vasculogenic mimicry.

Based on our current observations, we cannot confirm that AMPs have a significant role in the pathogenesis and progression of melanomas, but certainly they do not appear to influence major tumour characteristics associated with tumour progression in the cell lines analyzed. Given that melanomas often contain other AMP expressing cells, such as lymphocytes and macrophages[46], the lack of melanoma cell response to the tumour promoting effects of AMPs may be beneficial. Furthermore, AMPs may still find a place in the treatment of melanomas as their anti-cancer therapeutic properties and potential become better delineated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression of AMPs in uveal and cutaneous melanoma cell lines, and primary uveal melanoma samples expressed as fold change compared to primary cultured uveal melanocytes. Values represent data from 3 different passages for cell lines, and triplicates for primary tissues. Ribosomal Protein L27 (RPL27) was used as the reference gene. NE = not expressed. RNA samples used for human tissue controls were skin for hBD-1, hBD-2, hBD-3, testis for hBD-4, and bone marrow for LL-37 and HNP-1 (data provided in Supplementary Table 2). The complete data set for these experiments expressed as Cq values is shown in Supplementary Table 3.

A.

B.

Figure 2.

AMPs and melanoma cell migration. (A) Representative light microscopy images showing migration of OCM8 uveal melanoma cells across a scratch wound for up to 48 hours. At time 0 hours, a clean, well-defined wound edge was initially appreciated. 12 hours after the scratch, the cells started to migrate across the wound causing the wound edge to become irregular. At 24 hours, more cells are appreciated populating the denuded area, such that by 48 hours the wound edge became increasingly difficult to identify. Scale bars = 150µm. Images are representative of 3 independent experiments. (B) Quantitative representation of

cell migration shows that media with 10% FBS caused faster wound closure over time, whereas the AMPs LL-37 and HNP-1 do not appear to affect cell migration in SP6.5 and OCM8 melanoma cells. Data are the mean of three independent experiments. Error bars omitted due to the overlapping nature of the data.

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Figure 3.

Vasculogenic mimicry in cutaneous melanoma 3D cultures by brightfield microscopy. (A) Highly aggressive MUM2b cells grown in a 3D matrix for 3 days showed formation of looping patterns characteristic of vasculogenic mimicry. (B) Higher magnification showed cells arranged to form empty loops (stars). (C) After 17 days of growth in a 3D matrix, MUM2b cells have populated the loops and channels (arrows) forming the lining of the loops. (D) Less aggressive OCM8 cells at day 3 did not exhibit formation of looping patterns when grown on similar conditions. Scale bars = 150 μ m for A, 90 μ m for B, C, and D. Images are representative of 3 experiments.

Figure 4.

Fluorescence microscopy showing vasculogenic mimicry formation in 3D cultures of cutaneous melanoma. (A) Fluorescence imaging highlights the channel formed between two loop patterns running longitudinally. A larger chiasm could be seen at the center of the channel (arrow). (B) Higher magnification of the channel formed suggests patency of the hollow passageway. Note the bifurcation of the channel at the bottom of the image (arrow). (C) Channel surrounding a loop pattern seen anastomosing (arrow) with other segments of the vasculogenic network. MUM2b cells were stained with DAPI (blue), and Calcein AM (green) to highlight the cytoplasm. Scale bars = 90µm for A, C, 40µm for B. Images are representative of 3 experiments.

Figure 5.

AMPs and vasculogenic mimicry in cutaneous melanoma. Light microscopy images of MUM2b cutaneous melanoma cells grown in Matrigel 3D matrixes for up to 21 days, showed untreated MUM2b cells arranged to form looping patterns with cores devoid of cells as early as a few days after plating followed by progressive population of the loops over 2–3 weeks. Cells treated with LL-37 at 5 μ g/ml showed dissolution of the looping patterns at day 21, unlike untreated cells or cells treated with LL-37 at 10 μ g/ml. Treatment of cells with HNP-1 at 17.5 μ g/ml seemed to caused an increase in the proliferation of the cells, though formation of the loops was not abrogated. Scale bar is 150 μ m for all panels. Images are representative of 2 independent experiments.